Diagnosis and control of Mycoplasma bovis and Mycoplasma mycoides subspecies Mycoides small colony in cattle

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DIAGNOSIS AND CONTROL OF

MYCOPLASMA BOVIS AND

MYCOPLASMA MYCOIDES SUBSPECIES *MYCOIDES* SMALL COLONY IN CATTLE

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DEDICATION

I dedicate this thesis to my family

To my wonderful wife Helen, my sons Alex and Mark

To my mum, and to my dad who I miss so much

In memory of

Roger Miles

At rest 25th October 2001

ABSTRACT

Mycoplasmas are responsible for many important diseases of animals, including contagious bovine pleuropneumonia (CBPP) caused by *Mycoplasma mycoides* subsp. *mycoides* SC and calf pneumonia, arthritis and mastitis caused by *Mycoplasma bovis.* However, diagnostic techniques currently available are laborious and imprecise. The work described in this thesis concentrates on the critical evaluation of existing techniques and the development of improved procedures. The role of antimicrobial resistance in limiting the options for disease control was also considered.

Diagnostic methods for the detection of *M. mycoides* subsp. *mycoides* SC in clinical material were critically evaluated during a CBPP outbreak in Portugal. Immunoblotting was more sensitive and specific than CFT or ELISA. The polymerase chain reaction (PCR) was more rapid and sensitive than culture. However immunocytohistochemistry (ICC) was far the best test for detecting *M. mycoides* subsp. *mycoides* SC antigen in lungs.

A rapid latex agglutination test (LAT) to detect CBPP using a carbohydrate extract of *M. mycoides* subsp. *mycoides* SC was developed. Analysis of the carbohydrate extract composition demonstrated that fucose, glucosamine and galactose are present in the ratio of 1:2:16 respectively. *N*-acetyl neuraminic acid was also detected. Evaluation of the LAT with sera from negative, naturally infected and experimentally infected cattle demonstrates that the test clearly differentiates positive and negative CBPP sera. The LAT compared favourably with the CFT but was not as specific as the immunoblotting; however the LAT had the advantage of being more rapid and robust and could be used in the field.

Molecular methods including the polymerase chain reaction (PCR) and 16S rRNA gene sequencing were assessed for their potential use in the diagnostic laboratory. A PCR method for identifying *M. bovis* was adapted, evaluated and introduced as a routine laboratory test. Using a set of universal 16S rRNA gene primers, amplicons of two serologically untypable isolates, one from a peregrine falcon, and the other from an ostrich were obtained. Results imply that the isolates may be new mycoplasma species.

The development of antimicrobial resistance has been seen in many microorganisms but little evidence exists for resistance in mycoplasmas. Consequently, the *in vitro* effect of five antimicrobials; danofloxacin, oxytetracycline, spectinomycin, florfenicol and tilmicosin on 62 isolates of *M. bovis* and 20 of *M. mycoides* subsp. *mycoides* SC was investigated. Minimum inhibitory concentrations (MICs) and mycoplasmacidal (MMC) values were determined. Evidence of antimicrobial resistance by *M. bovis* is shown. The potential for *M. mycoides* subsp. *mycoides* SC to develop antimicrobial resistance against spectinomycin *in vitro* is also demonstrated.

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LIST OF ABBREVIATIONS

A.	*Acholeplasma*
Å	Angstrom unit (10^{-10}metre)
ABC	Avidin-biotin-peroxidase complex
AIDS	Autoimmune deficiency syndrome
ALF	Automated laser fluorescent
ATP	Adenine triphosphate
BSA	Bovine serum albumin
C	Cytosine
cfu	Colony forming units
CA	Contagious agalactia
CBPP	Contagious bovine pleuropneumonia
CCPP	Contagious caprine pleuropneumonia
CFT	Complement fixation test
CO_2	Carbon dioxide
DEFRA	Department of Environment, Food and Rural Affairs
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbant assay
EMP	Embden Meyerhof-Parnas
EMPRES	Emergency prevention system
FAO	Food and Agriculture Organization (of United Nations)
DFIT	Disc film inhibition test
G	Guanine
GIT	Growth Inhibition Test
H.	*Haemophilus*
HMS	Hexose monophosphate shunt
HPLC	High performance liquid chromatography
ICC	Immunocytohistochemistry
Ig	Immunoglobulin
IFAT	Indirect fluorescent antibody test
IHA	Indirect haemagglutination assay
IS	Insertion sequence
ISCOM	Immunostimulating complex
kb	Kilobase
kDa	Kilodalton
LAT	Latex agglutination test
LC	Large colony
M.	*Mycoplasma*
mA	Milliamp
Mab	Monoclonal antibody
MAFF	Ministry of Agriculture, Fisheries and Food, replaced by DEFRA
MIC	Minimum inhibition concentration
MLO	Mycoplasma like organism

nm	Nanometres
OIE	Office International des Epizooties
ORF	Open reading frame
p	Pico
PBS	Phosphate buffered saline
PFGE	Pulsed field gel electrophoresis
PHA	Passive haemagglutination assay
PPLO	Pleuropneumonia-like organism
REA	Restriction enzyme analysis
rrn	Ribosomal ribonucleic acid operon
rRNA	Ribosomal ribonucleic acid
RSAT	Rapid slide agglutination test
S	Svedberg units
SC	Small colony
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
subsp.	Subspecies
Taq	*Thermus aquaticus*
TCA	Tricarboxylic acid
Th 1	T helper cells
TLC	Thin Layer Chromatography
TMB	3,3',5,5'-tetramethylbenzidene
μm	Micrometres
VLA	Veterinary Laboratories Agency
vsp	Variable surface protein
W	Watt
w/v	Weight to volume

CHAPTER 1

1. 1. INTRODUCTION

1. 1. 1. MOLLICUTES

Within the Kingdom *Procaryotae,* there are four divisions (Razin and Freundt, 1984): *Firmicutes,* the gram positive bacteria; *Gracilicutes,* the gram negative bacteria; *Mendosicutes,* the archaebacteria; and, *Tenericutes,* the cell wall-less bacteria. *Mollicutes* is the sole class in this division and contains the genera, *Ureaplasma, Spiroplasma, Acholeplasma, Anaeroplasma, Asteroleplasma, Entomoplasma, Mesoplasma* and *Mycoplasma.* The genera *Mycoplasma* and *Ureaplasma* form the family *Mycoplasmataceae* (Razin *et al.,* 1998).

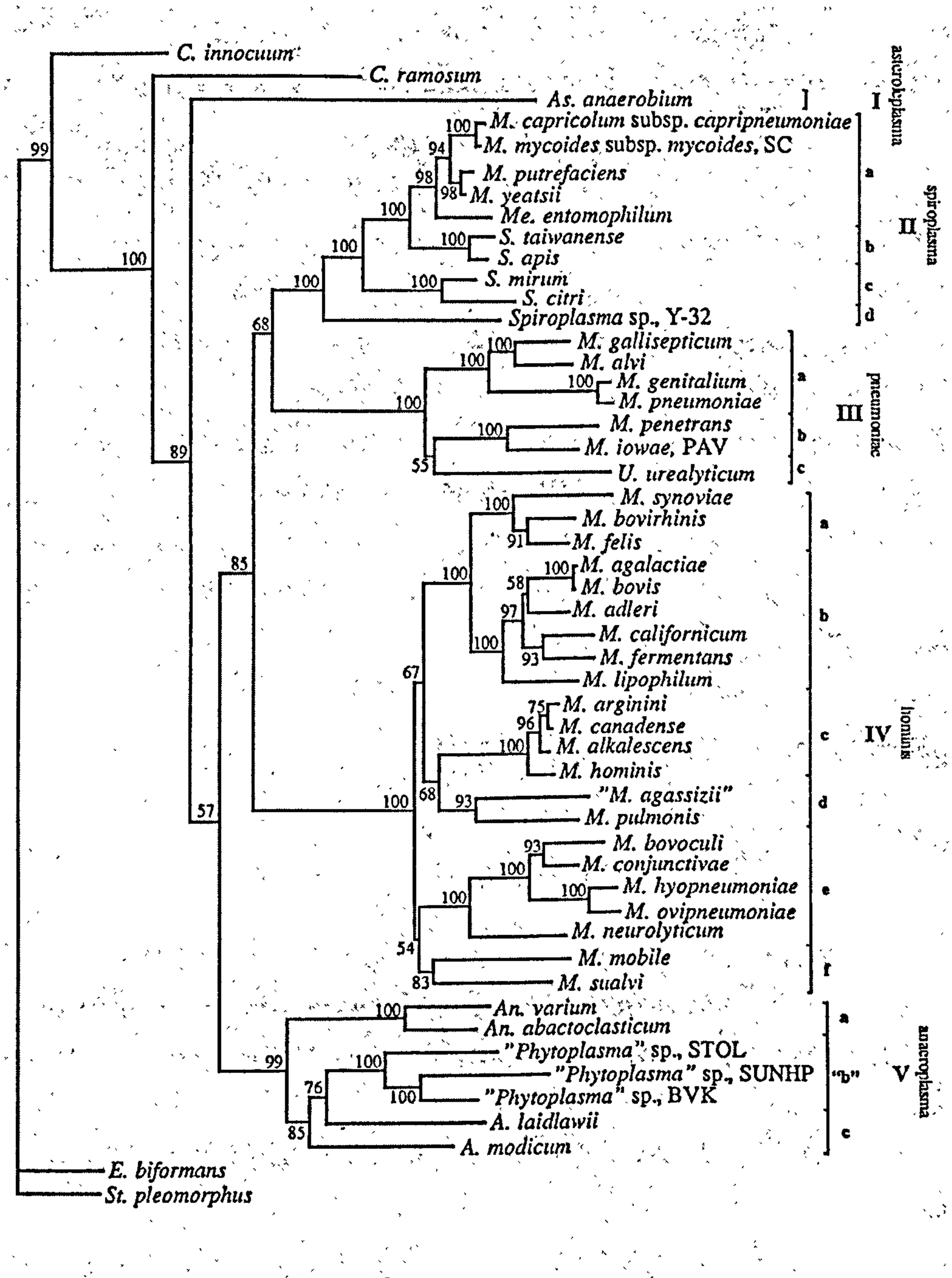
1. 1. 2. MAJOR CHARACTERISTICS OF MOLLICUTES

Mollicutes are prokaryotic organisms that are generally smaller than other bacteria and lack a cell wall, which increases their susceptibility to osmotic stress. The absence of specific cell wall associated polymers also renders mollicutes resistant to the action of antimicrobials (such as penicillin and cycloserine) which act against cell wall synthesis (Rosenbusch, 1994). Cell size ranges from 0.15 μm to over 1 μm in diameter and the small size and plasticity of cells enables them to pass through 0.45 μm bacteriological filters. Generally, *Mollicutes* can be cultivated on cell-free nutrient media enriched with serum that provides sterols and fatty acids. On agar media they give a characteristic 'fried egg' colonial morphology caused by the cells in the central zone of the colony growing into the medium (Razin and Freundt, 1984).

The genera *Asteroleplasma, Acholeplasma, Anaeroplasma, Mycoplasma* and *Ureaplasma* are mostly found in man or animals, but one species, *A. laidlawii,* has also been isolated from soil and sewage, although it does not grow in these environments. *Spiroplasma* and *Mesoplasma* occur in plants and insects with *Entomoplasma* having only been isolated from insects. Both *Mesoplasma* and *Entomoplasma* have been classified as belonging to the *mycoides* phylogenetic cluster within the spiroplasma phylogenetic group (Dybvig and Voelker, 1996; see Figure 1.1.). The first mollicute to be isolated, which became the type species of

Mycoplasma, was isolated from cattle affected by contagious bovine pleuropneumonia (Nocard and Roux, 1898); it was later named *Mycoplasma mycoides* (Edward and Freundt, 1956). Mycoplasmas subsequently isolated were called pleuropneumonia like organisms (PPLO's).

FIGURE 1. 1. PHYLOGENY OF MYCOPLASMAS BASED ON THE 16S rRNA GENES



10.

Numbers represent % relatedeness based on distance matrix analysis. Bar shows 10% value.

Figure adapted from information supplied by Pettersson *et al.* (1999).

The major characteristics of mollicutes are described in Table 1.1. The mollicutes are characterised by the low guanine plus cytosine (GC) content of their DNA (23-40 %). They possess a circular double-stranded DNA chromosome of ~600 kb to ~1,800 kb and include the organisms with the smallest known genome size. The genome sizes of some mollicutes are compared with other bacteria in Table 1. 2.

Because of their small genome size and simple ultrastructure, mollicutes, in particular mycoplasmas, have attracted significant attention in the search to determine the key components required for life and for the 'minimal cell' (Wells, 1997). Proposals were first made in 1984 to carry out the complete physical and functional mapping of a mycoplasma genome. This was to include determination of: the entire DNA sequence; the open reading frames (ORFs) present; and the amino acid sequences encoded so defining all the genes of the mycoplasma and their products. In this way, a complete molecular description of the machinery of a cell would be achieved (Morowitz, 1984). Since 1984 many genomes have been sequenced, including those of *M. genitalium, M. pneumoniae, M. pulmonis* (Chambaud *et al.*, 2000) and *Ureaplasma urealyticum* (Glass *et al.*, 2000) and the cell-walled bacteria *Haemophilus influenzae* and *Escherichia coli* (Razin *et al.*, 1998). Complete genome sequences for 30 microbial species have been determined and a further 100 are expected in the next four years (Fraser *et al.*, 2000). A comparison of the properties of selected bacterial genomes is given in Table 1. 3. The availability of complete genome sequences has enhanced comparative studies of the functional contents of genomes and their evolution. One finding demonstrates that the absence of a cell wall in *Mycoplasma* leads to a significant saving in the genetic information required by cells. The synthesis of cytoplasmic membrane proteins in *M. genitalium* and *M. pneumoniae* requires only 30 and 54 genes respectively. In contrast, 105 genes have been identified which are involved in cell envelope synthesis in the gram-negative *H. influenzae* (Razin *et al.*, 1998).

TABLE 1. 1. THE MAJOR CHARACTERISTICS AND TAXONOMY OF THE CLASS *MOLLICUTES*

Taxa	Number of recognized species (1998)	Genome Size (kb)	MOL% G+C OF GENOME	Cholesterol requirement	Distinctive properties	Habitat
Order I: *Mycoplasmatales*						
Family I *Mycoplasmataceae*						
Genus I: *Mycoplasma*	102	580-1,350	23-40	Yes	Optimum growth at 37°C	Humans, animals
Genus II: *Ureaplasma*	6	760-1,170	27-30	Yes	Urea hydrolysis	Humans, animals
Order II: *Entomoplasmatales*						
Family I *Entoplasmataceae*						
Genus I: *Entomoplasma*	5	790-1,140	27-29	Yes	Optimum growth at 30°C	Insects, plants
Genus II: *Mesoplasma*	12	870-1,100	27-30	No	Optimum growth at 30°C; 0.04% Tween 80 required in serum-free medium	Insects, plants
Family II *Spiroplasmataceae*						
Genus I: *Spiroplasma*	33	780-2,220	24-31	Yes	Helical motile filaments; Optimum growth at 30-37°C	Insects, plants
Order III: *Acholeplasmatales*						
Family I *Acholeplasmataceae*						
Genus I: *Acholeplasma*	13	1,500-1,650	26-36	No	Optimum growth at 30-37°C	Animals, also for some species plants, insects
Order IV:						
Family I *Anaeroplasmataceae*						
Genus I: *Anaeroplasma*	4	1,500-1,600	29-34	Yes	Oxygen-sensitive anaerobes	Bovine/ovine rumen
Genus II: *Asteroleplasma*	1	1,500	40	No	Oxygen-sensitive anaerobes	Bovine/ovine rumen
Undefined taxonomic status						
Phytoplasma	ND[a]	640-1,185	23-29	Not known	Uncultured *in vitro*	Insects, plants

[a] Taxonomic status not yet defined; two *Candidatus* Phytoplasma species have been published (Davis *et al.*,1997; Zreik *et al.*, 1995).

Table adapted from Razin *et al.* (1998).

TABLE 1. 2. COMPARISON OF GENOME SIZES IN MOLLICUTES AND OTHER BACTERIAL SPECIES

Species	Size kb
Mycoplasma genitalium	580
Mycoplasma hominis	684
Ureaplasma urealyticum serovar 3	752
Mycoplasma mobile	780
Mycoplasma pneumoniae	816
Mycoplasma synoviae WVU 1853	900
Mycoplasma gallisepticum PG31	1,050
Mycoplasma hyopneumoniae strain J	1,140
Mycoplasma fermentans	1,160
Mycoplasma mycoides subsp. *mycoides* Y	1,240
Mycoplasma iowae	1,280
Mycoplasma mycoides subsp. *mycoides* GC 1176-2	1,330
Acholeplasma laidlawii	1,580
Helicobacter pylori	1,660
Haemophilus influenzae	1,830
Vibrio cholerae El Tor N1696	4,030
Escherichia coli K-12	4,639
Bacillus cereus	5,700
Pseudomonas aeruginosa	5,900

Data obtained from: Krawiec and Riley (1990); Barlev and Borchsenius (1991); Fraser *et al.* (1995; 2000); Glass *et al.* (2000); Himmelreich *et al.* (1996); Razin *et al.* (1998).

TABLE 1. 3. GROSS PROPERTIES OF SOME BACTERIAL GENOMES

Property	***M. genitalium***	***M. pneumoniae***	***H. infuenzae***	***E. coli***
No. of bp	580,070	816,394	1,830,137	4,639,221
G+C content (mol%)	32	40	38	50
No. of putative coding sequences (ORFs)	479	677	1,703	4,288
No. of ORFs tentatively identified	468	603	1,408	2,659
No. of ORFs with no functional prediction or database match	11	74	295	1,629

Table adapted from Razin *et al.* (1998).

1. 1. 3. PHYLOGENY

Mycoplasma genitalium has the smallest genome of any known organism capable of independent replication with just 580,070 base pairs (Fraser *et al.*, 1995). This may be close to the minimal genome size necessary for cellular survival. Two theories have been proposed as to how mollicutes with such small genomes have evolved. Wallace and Morowitz (1973) considered mollicutes as extremely primitive living relics that preceded present day bacteria, whereas others saw them as degenerate forms of what might be called "normal" bacteria (Razin, 1978). If the latter theory is correct, the genome size of mollicutes must have been severely reduced during their evolution, with only the essential functions being retained, leading to a reliance on their hosts for nutrients.

The study of evolution using DNA composition and immunological relationships provides little phylogenetic information, although it is useful for taxonomy. However, the examination of highly conserved ribosomal RNA (rRNA) has provided much information on evolution. The longer 16S rRNA gene is more

useful than the smaller 5S rRNA gene, allowing accurate determination of phylogenetic distance (Woese, 1987).

On the basis of sequence information it is now accepted that the class *Mollicutes* constitutes a phylogenetically distinct group of highly unusual bacteria that are only distantly related to other eubacteria (Gibbons and Murray, 1978; Razin, 1978, Kamla *et al.*, 1996). They appear to have evolved from clostridia, with cell wall and other genes being lost during evolution. Using a hypothetical model for mollicute phylogeny, Maniloff (1996) concluded that the ancestral mollicute arose from the *Streptococcus* phylogenetic branch about 600 million years ago, probably from an organism with a genome of 2,000 kb. Degenerate evolution continued, giving the different genera within the class *Mollicutes* that currently exist.

Although mutation rates have not been widely measured, mollicute rRNA sequences appear to change more rapidly than those of other eubacteria. Thus, there are significant variations amongst the mollicutes sequences (Woese *et al.*, 1985), even within well-defined species. In *M. gallisepticum* for example, bases at generally conserved positions are known to vary (Woese, 1987). However, it has been shown that mycoplasmas lack DNA polymerase 3'-5' exonuclease activity. This may limit their ability to repair genetic errors, so leading to high mutation rates (Woese, 1987).

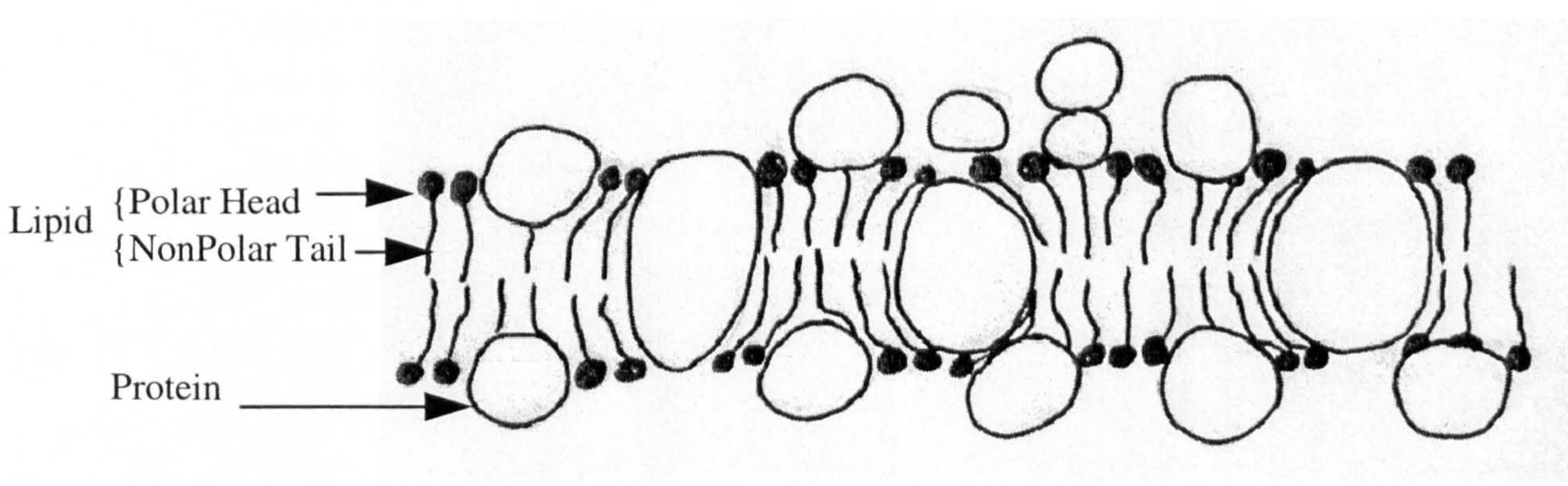
The phylogenetic relationships amongst mollicutes, based on 16S rRNA homology are given in Figure 1.1. Five major groups are recognised: asteroleplasma, spiroplasma, pneumoniae, hominis and anaeroplasma. Within these groups organisms that are very closely related, based on the 16S rRNA sequences, are grouped in clusters.

1. 2. STRUCTURE, METABOLISM AND PATHOGENICITY

1. 2. 1. THE CELL ENVELOPE AND CYTOSKELETON

The most notable differences between mollicutes and other prokaryotes are the inability of mollicutes to synthesise a cell wall and their lack of intracellular membrane structures. Direct proof for the absence of cell walls was obtained by electron microscopy of thin sections of mycoplasma cells (van Iterson and Ruys, 1960; Domermuth *et al.*, 1964). Osmium-fixed sections showed that cells were bounded by a single trilaminar membrane, about 8-10nm thick. Proteins constituted over two-thirds of mollicute membrane mass, with the rest being membrane lipids (Razin *et al.*, 1998). Studies on the molecular organization and function of the lipids in mollicute membranes have been aided by the dependence of *Mollicute* species on external supplies of fatty acids and sterol. By varying the nature and concentration of fatty acids and sterol in the growth medium, alterations in membrane lipid composition may be achieved (Razin *et al.*, 1998). The bulk of the membrane lipids form a bilayer (Steim *et al.*, 1969), in which the distribution of phospholipids and glycolipids shows a definite transbilayer asymmetry (Razin, 1993). Mollicutes alter the polar head group structure of membrane and phospho- and glycolipids, and change the acyl chain structure, in response to changes in environmental and physiological conditions (Andersson *et al.*, 1996); the most likely membrane structure for *Mycoplasma* species is shown in Figures 1.2 and 1.3 (Razin, 1993). Mycoplasmas typically have a number of distinct lipoproteins anchored on the outer face of the plasma membrane (Figure 1.3; Chambaud *et al.*, 1999). These surface antigens are preferential targets for the host immune response. However, variation of their size and expression provides mycoplasmas with an effective means of evading the host immune defence system. The plasma membranes of some *Mycoplasma* species also contain lipoglycans, which although clearly distinct from the lipopolysaccharides of the outer-membrane of Gram –ve bacteria, similarly possess a lipid membrane anchor and a repetitive oligosaccharide structure (Fig. 1.3; Chambaud *et al.*, 1999).

FIGURE 1. 2. THE ORGANISATION OF PROTEINS AND LIPIDS IN MOLLICUTE MEMBRANES



Model showing the most likely arrangement of proteins and lipids in the molecular organisation of mollicute membranes.

Figure adapted from Razin (1993).

FIGURE 1. 3. THE MOLECULAR ORGANIZATION OF THE MYCOPLASMA CELL ENVELOPE

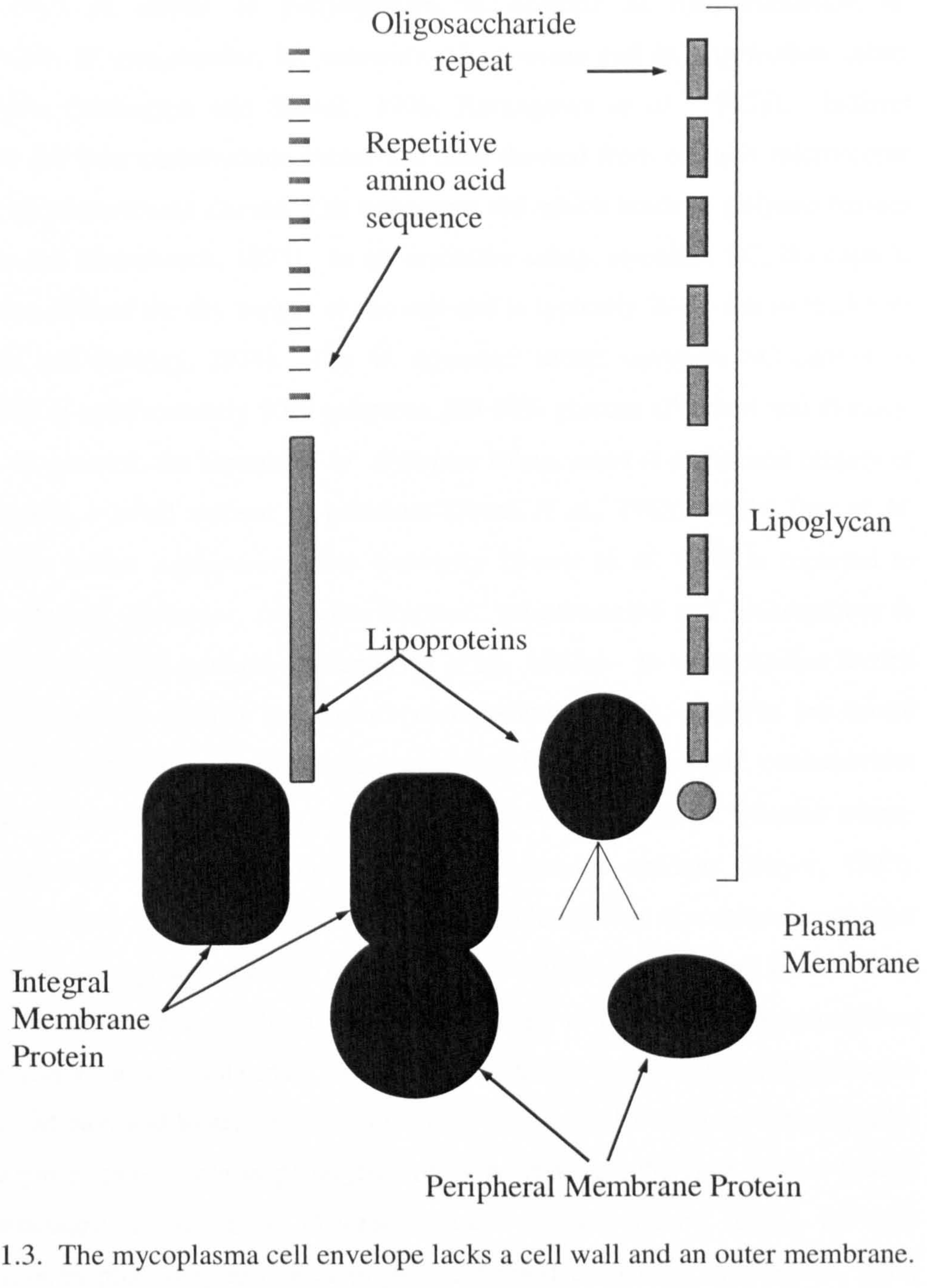


Figure 1.3. The mycoplasma cell envelope lacks a cell wall and an outer membrane. In mycoplasmas, lipoproteins are anchored onto the outer surface of the plasma membrane.

Figure adapted from Chambaud *et al.* (1999).

Capsules are bound to the outside of the cell membrane in some *Mycoplasma* and *Ureaplasma* species, including *M. mycoides* subsp. *mycoides* small colony (SC), *M. dispar, M. gallisepticum, M. hominis, M. hyopneumoniae, M. meleagridis, M. pneumoniae, M. pulmonis, M. synoviae* and *M. capricolum* subsp. *capricolum* (Robertson and Smook, 1976; Rurangirwa *et al.,* 1987a). Indirect evidence for their carbohydrate nature has been derived from electron microscopic studies on preparations stained with ruthenium red which binds to polysaccharides (Minion and Rosenbusch, 1993). In *M. mycoides* subsp. *mycoides* SC, the capsule comprises 10% of the dry weight of the cell and is typically 20-40 nm in thickness (Howard and Gourlay, 1974). The *M. mycoides* subsp. *mycoides* SC capsule is composed of approximately 90% galactose and 10% glucose (Plackett and Buttery, 1958). In contrast, the capsule of *M. mycoides* subsp. *capri* is composed largely of glucose with a small amount of galactose (Jones *et al.,* 1965), whilst that of *M. capricolum* subsp. *capripneumoniae* (formerly known as *M.* F38) is reported to contain glucose, galactose, mannose, fructose, galactosamine and glucosamine in approximately equal amounts (Rurangirwa *et al.,* 1987a). In some studies lectins have been used to identify cell-surface carbohydrate groups. Lectins are useful because they are highly specific in the groups they bind. For example, concanavalin *A* binds to internal and nonreducing terminal α-mannosyl residues, whereas wheat-germ agglutinin binds to terminal *N*-acetylglucosamine residues (Stryer, 1995). Studies based on lectin binding to cells have concluded that mycoplasma capsules are generally composed of acidic carbohydrates or lipids (Minion and Rosenbusch, 1993). Capsules have not been described amongst acholeplasmas, spiroplasmas, anaeroplasmas or asteroplasmas. Mycoplasma capsule function generally remains obscure (Minion and Rosenbusch, 1993). However, in cell-walled bacteria capsules are thought to play a role in pathogenicity by promoting adherence to host tissues and particularly to negatively charged receptor surfaces (Robb, 1984), by their toxicity, or by promoting resistance to phagocytosis (Rosenbusch and Minion, 1992; Marshall *et al.,* 1995).

The anti-phagocytic properties of polysaccharide capsules are associated with their electronegative charge, the prevention of the nonspecific deposition of immunoglobulin G (Absolom, 1988), and the capacity to bind non-specific factor H,

an inhibitor of the alternative pathway of complement activation (Kasper, 1986). Additional protection is also provided where cell aggregates are surrounded by a common capsule (glycocalyx) of exopolysaccharide material. This increases resistance to degradative enzymes (Isenberg, 1998), but it is not clear whether such aggregates are commonly formed by mollicute cells. In mollicutes, the importance of the capsule in resisting phagocytosis was demonstrated by Marshall *et al.*, (1995). They showed that mutant strains of *M. mycoides* subsp *mycoides* which produced reduced quantities of capsular polysaccharide, had an increased susceptibility to phagocytosis in an *in vitro* assay.

Association of the mycoplasma capsule with attachment was first suggested by Wilson and Collier (1976). Attachment of *M. dispar* to erythrocytes appeared to be mediated by ruthenium red-stainable capsular material. However, other possible methods of attachment in the form of fine extracellular threads or fibrils were previously observed between the mycoplasma membranes and erythrocytes by Howard and Gourlay, (1974). However, strains of *M. hyopneumoniae* that were extensively passaged *in vitro* did not exhibit fibrillar structures and were less pathogenic to pigs (Tajima and Yagihashi, 1982), indicating attachment mechanisms have an effect on the pathogenicity of mycoplasmas.

In addition to their roles in attachment and avoiding phagocytosis, mycoplasma capsules may have a direct toxic effect on host cells. Calves inoculated intravenously with the purified galactan capsule of *M. mycoides* subsp. *mycoides* SC, showed increased mycoplasmaemia and polyarthritis when subsequently challenged with viable organisms (Lloyd *et al.*, 1971). Another effect ascribed to the galactan of *M. mycoides* subsp. *mycoides* was that it promoted deposition of fibrin around chronic lung lesions in cattle (Buttery *et al.*, 1980); it also increased their sensitivity to subsequent challenge with live organisms (Buttery *et al.*, 1976). The galactan capsule has a close biochemical relationship with that of the host lung tissue pneumogalactan and might therefore trigger an auto-immune reaction (Ferronha *et al.*, 1988).

Recently, capsular carbohydrates have received increasing attention and have been investigated for use in vaccines (Abusugra and Morein, 2000); and in

ELISA and rapid latex-agglutination tests for antibodies in serum (see chapters 5 and 6) (Ayling *et al.*, 1999; March *et al.*, 1999)

Mollicute cells are generally described as pleomorphic; they vary in shape from spherical or pear-shaped structures to branched or helical filaments. Genome replication precedes, but is not necessarily synchronised with cell division (Razin, 1995). Thus, budding forms and filaments are frequently seen, especially in species such as *M. mycoides.* Although mollicutes have no flagella and are mostly non-motile, some mycoplasmas exhibit gliding motility on liquid-covered surfaces. These gliding mycoplasmas generally grow as pear-shaped cells with a specialised tip structure. Studies on the *M. pneumoniae* tip structure show that it plays an important role in colonisation of host cell surfaces by providing orientation and polarity of attachment (Baseman, 2000). A protein identified as P1 is localised in the tip region and is probably the major component responsible for attachment (Carson *et al.*, 1992) although there is evidence of other mechanisms of adherence and colonization (Baseman, 2000). The tip structure consists of a striated rod with associated microfilaments, possibly representing a primitive cytoskeleton (Razin, 1995). Proteins related to P1 have been identified in *M. genitalium* (Tully *et al.*, 1983), *M. gallisepticum* (Athamna *et al.*, 1997) and a range of other human and animal mycoplasmas.

1. 2. 2. METABOLISM

The small genome size of mollicutes limits the range of their metabolic activities and they are largely dependent on extracellular sources of amino acids, nucleic acid precursors and lipids. In typical mycoplasma media, these are contained within such components as serum, yeast and animal tissue extracts and protein digests (see Chapter 2, Section 2). As the precursors for synthetic pathways need to be provided in the environment, the role of catabolic pathways is mainly concerned with energy generation (Miles, 1992). In the majority of mollicutes, glucose and other metabolizable polysaccharides are the sole or major energy sources. In addition, certain *Mycoplasma* and *Spiroplasma* species are able to generate ATP from the hydrolysis of arginine (Pollack, 1979) and in *Ureaplasma*, energy generation is dependent on urea hydrolysis (Masover *et al.*, 1977; Glass *et al.*, 2000).

In *M. mycoides* glucose uptake is by a phosphoenol pyruvate:phosphotransferase system (PEP:PTS) (Cirillo, 1979) and metabolism proceeds by the Embden-Meyerhof pathway (Cocks *et al.*, 1985). Anaerobically, lactate is the end product of glucose metabolism and there is a net yield of 2 mol ATP/mol glucose. However, under aerobic conditions, there is a partial oxidation and pyruvate is converted *via* phosphate-acetyl transferase and acetate kinase activities to acetate plus CO_2, giving a total of 4 mol ATP/mol glucose. Mollicutes lack the tricarboxylic acid cycle (TCA) and acetate is not further metabolised.

Where pyruvate is oxidised to acetate, there is a net production of NADH, which is reoxidised to NAD^+ by molecular oxygen through the action of NADH oxidase. Cocks *et al.* (1985) assumed the product of NADH oxidation to be H_2O_2. However, Miles *et al.* (1991) and Taylor *et al.* (1996) reported that water was the major product of NADH oxidation in the *Mycoplasma* species they studied. Subsequently, Rice *et al.* (2001) showed that in *M. mycoides* subsp. *mycoides* strains, the quantity of H_2O_2 produced varied. This is potentially significant in pathogenicity since H_2O_2 may cause host cell damage.

In fermentative mycoplasmas, sugars other than glucose may sometimes be utilised and patterns of substrate utilization are potentially useful in the characterisation of species (Miles *et al.*, 1991; Abu-Groun, *et al.*, 1994; Taylor *et al.*, 1996). For example, maltose and trehalose were metabolised by strains of *M. mycoides* subsp. *capri* (Wadher and Miles, 1988) and certain other members of the "*M. mycoides* cluster" (Abu-Groun *et al.*, 1994), but not by *M. mycoides* subsp. *mycoides* SC strains (Miles & Lee, 1983; Abu-Groun *et al.*, 1994).

Mycoplasma mycoides may be considered typical of those species that produce acid from glucose; however in a number of other glucose fermenting *Mycoplasma* species, including *M. fermentans* and *M. canis*, pyruvate is not oxidised to acetate and lactate appears to be the end product of glucose metabolism under aerobic and anaerobic conditions (Miles *et al.*, 1991). In contrast, some non-fermentative mycoplasmas are able to oxidise pyruvate and in some of these species, organic acid oxidation appears to be the sole source of ATP generation. Examples of these species include *M. agalactiae* and *M. bovis* (Miles *et al.*, 1994; 1996).

1. 2. 3. PATHOGENICITY

Most mollicutes exist as commensals, apparently living in harmony with their host (Razin *et al.*, 1998). However, many *Mycoplasma* species are pathogenic (see Section 1.3). In many cases it appears that the major symptoms of disease result from damage due to host immune and inflammatory responses rather than to direct effects of the mycoplasmas themselves (Razin *et al.*, 1998). Host immune responses are generally brought about by the induction of cytokine synthesis and are outside the scope of this thesis. Other factors which are important or potentially important in pathogenicity include: adhesion to host cell surfaces; the ability to avoid host defences by an intracellular location (Baseman, 2000) or the ability to penetrate cell crypts; the production of a capsule (see Section 1.2.1); the production of toxins; the depletion of host cell substrates, particularly of arginine by arginine-hydrolysing mycoplasmas; the production of mitogens which cause non-specific stimulation and proliferation of lymphocytes (Naot, 1995); surface antigenic variation; and the secretion of degradative enzymes. Those factors of particular importance to pathogenicity in *M. mycoides* and/or *M. bovis* are considered below.

The adhesion of mollicutes to host cells is a prerequisite for colonisation and for infection. Amongst mycoplasmas, the most detailed knowledge of adhesion mechanisms has been obtained for *M. pneumoniae*. In this organism, binding to solid surfaces and animal cells requires the P1 adhesin together with a number of accessory proteins which are concentrated within the cell membrane of a tip organelle (see Section 1.2.1.; Krause, 1996, 1998); the correct formation of the P1 structure is a prerequisite for a successful infection (Krause, 1998). The adhesion mechanism of *M. gallisepticum* also involves a specialised tip-like organelle and appears similarly complex (Athamna *et al.*, 1997). Adhesion is dependent upon a PvpA surface protein, which has a high homology to other mycoplasmal adhesins and shares an epitope with *M. bovis* (Yogev *et al.*, 2000). Sachse *et al.* (1996) studied cytadhesion of *M. bovis* to embryonic bovine lung cells and concluded that adherence rates were strongly dependent on temperature and involved variable surface proteins (vsps). Following adhesion to host cells most mycoplasmas appear to remain surface-attached; however, evidence of tissue invasion and an intracellular location in certain species is accumulating, notably for *M. bovis, M. fermentans, M.*

penetrans and *M. gallisepticum* (Howard *et al.*, 1987; Pfutzner, 1990; Lo, S-C, 1992, Winner *et al.*, 2000).

The toxic by-products of mycoplasma metabolism, such as hydrogen peroxide and superoxide radicals have been incriminated in causing oxidative damage to host cell membranes (Razin, 1991; Houshaymi *et al.*, 1997). Hydrogen peroxide may be formed during the oxidation of NADH (see Section 1.5.3.); however, it is also formed in large quantities during the oxidation of a-glycerophosphate, an intermediate in the metabolism of glycerol (Taylor *et al.*, 1996). All *M. mycoides* strains (except European *M. mycoides* subsp. *mycoides* SC strains) oxidise glycerol at high rates (Rice *et al.*, 2001). There is also evidence that some mycoplasmas produce high molecular weight toxins. *M. neurolyticum* produces a membrane-associated toxin and some variants of *M. gallisepticum* produce a neurotoxin (Kumars *et al.*, 1963; Thomas, 1967). An inflammatory toxin has been extracted from *M. bovis* and toxic components have been described for other mycoplasmas including *M. arthritidis* and *M. hyopneumoniae.* The galactan capsule produced by *M. mycoides* may also have a pathogenic role, though the mechanism is poorly understood (Egwu *et al.*, 1996).

One means by which pathogens may evade detection by the host immune system is to vary their surface antigens. Surface antigenic variation in mycoplasmas was first discovered in *M. bovis* (Behrens *et al.*, 1994). In the *M. bovis* system, lipoproteins designated as vsps, undergo frequent and spontaneous changes in expression state and in size (Behrens *et al.*, 1994). More recently, surface antigenic variation has been demonstrated in *M. gallisepticum, M. agalactiae, M. pulmonis, M. hominis, M. fermentans* and *M. hyorhinis* (Razin *et al.*, 1998). In *M. bovis* variation is thought to be caused by the random transposition of an insertion element in the regulatory region of the *vspA* gene (Lysnyansky *et al.*, 1998).

There is little evidence to suggest that the secretions of degradative enzymes play a significant role in mycoplasma pathogenicity. However, proteolytic activity, as detected by casein hydrolysis or the liquefaction of inspissated animal serum or gelatin, is present in a number of species. In addition, *M. mycoides* subsp. *mycoides* SC strain Y appears to be able to obtain some amino acids for growth by the degradation of bovine serum albumin (Rodwell, 1983). Thus, it has been

suggested that mycoplasma protease activity may be involved in arthritic disease (Cole *et al.*, 1985; Czekalowski *et al.*, 1973). Many mycoplasma diseases manifest as arthritis in animals and experimental infections in cattle with *M. bovis* resulted in the destruction of cartilage and development of fibrotic lesions within joints (Simecka *et al.*, 1992).

1. 3. HABITAT AND ROLE IN DISEASE

1. 3. 1. MOLLICUTES IN ARTHROPODS AND PLANTS

A variety of mollicutes are found in arthropods and plants. These include the helical spiroplasmas, entomoplasmas, mesoplasmas, acholeplasmas, mycoplasmas and the mycoplasma-like organisms (MLOs) which are now referred to as phytoplasmas. Mycoplasmas and acholeplasmas, resembling those of classical animal species, were first reported on vegetable and floral plant surfaces in 1979 (Eden-Green and Tully, 1979; McCoy *et al.*, 1979).

Spiroplasmas have been isolated from plants that show symptoms of chlorosis, leaf mottling, proliferation of growing points, and general stunting. Some spiroplasmas are involved in vector-transmitted plant diseases such as citrus stubborn and corn stunt. These pass through a complex biological cycle that involves passage through an insect host before reinfection of healthy plants. Spiroplasmas have been reported in *Hymenoptera* (honey bees, wasps), *Coleoptera* (beetles), *Diptera* (flies, blood-sucking insects such as mosquitoes, tabanid flies, *Drosophila* spp), *Lepidoptera* (butterflies, which feed on flower nectar), *Homoptera* (leafhoppers) and *Hemiptera* (leaf bugs) (Hackett, 1990). They have also been isolated from ticks (Razin, 1992). The spiroplasmas are phylogenetically most closely related to the *M. mycoides* cluster based on 16S rRNA gene sequencing (Figure 1.1.).

Phytoplasmas, first discovered in the 1960's (Doi *et al.*, 1967), are the most economically important group amongst the plant mollicutes. More than 300 phytoplasma associated plant diseases transmitted by insects have been described (McCoy *et al.*, 1989). Little is known about these organisms as they have not yet been cultivated, but they are maintained artificially in plants by vegetative propagation methods, such as grafting, cuttage and tissue culture (Tanaka *et al.*,

2000). The recent application of molecular techniques has shown that they have many properties in common with classical mollicutes, including a small genome size and a low GC content. Lim and Sears (1991) demonstrated that *Phytoplasma* genome size was similar to that of *Mycoplasma* and smaller than in *Acholeplasma.* However, on the basis of 16S rDNA sequence homology, phytoplasma show a closer evolutionary relationship to *Acholeplasma* (Figure 1.1). Use of 16S rDNA sequencing amplification of the 16-23S rDNA intergenic region, has identified 11 major groups of phytoplasmas (Wang and Hiruki, 2000).

1. 3. 2. MOLLICUTES *IN MAN*

The first isolation of a mycoplasma from man, probably *M. hominis*, was in 1937 (Dienes and Edshall, 1937). By 1955 three mycoplasmas associated with humans had been discovered (*M. hominis, M. fermentans* and *M. salivarium*) and many others have since been isolated (Table 1.4.); some of these are considered to be part of the normal flora, but *M. pneumoniae* and a small number of additional species are associated with disease. These are described in the following sections (Section 1.3.2.1-1.3.2.2.).

TABLE 1. 4. *MOLLICUTES* ISOLATED FROM MAN

Species	Other hosts	Site of recovery and occurrence	Pathogenicity
Acholeplasma laidlawii	Birds, domestic animals, other primates	Oral cavity, respiratory and urogenital tracts; rare	Not known
Mycoplasma arthritidis	Rodents	Urogenital tract; only one report	Apparently non-pathogenic
Mycoplasma A39		Lung	Bronchitis in immunosupressed patients (Pitcher, 2001)
Mycoplasma buccale	Monkey	Oropharynx, vagina (monkeys); uncommon	Apparently non-pathogenic
Mycoplasma faucium	Other primates	Oropharynx; uncommon	Apparently non-pathogenic
Mycoplasma fermentans	Other primates	Urogenital tract, oropharynx; uncommon	Not known; see Section 1.3.2.3. produces chromosomal aberrations in cell culture and leukemoid disease in mice
Mycoplasma genitalium		Urogenital tract, rectum; uncommon	Associated with NGU and possibly pelvic inflammatory disease (Taylor-Robinson, 2001)
Mycoplasma hominis	Other primates	Urogenital tract; common	Pyelonephritis, pelvic inflammatory disease, postabortal/postpartum fever, arthritis, congenital pneumonia
Mycoplasma lipophilium		Oral cavity; rare	Not known
Mycoplasma orale	Other primates	Oropharynx, contaminated cell cultures; widespread	Apparently non-pathogenic
Mycoplasma pneumoniae		Respiratory tract, oral cavity; rarely middle-ear and spinal fluid	Cause of primary atypical pneumonia; other disease manifestations include bullous myringitis, myocarditis, cutaneous and neurological conditions
Mycoplasma primatum	Other primates	Urogenital tract; rare in man	Not-known
Mycoplasma salivarum	Other primates	Oral cavity; common	Apparently non-pathogenic
Ureaplasma urealyticum		Urine, semen, urethra, vagina, cervix, mouth, pharynx, rectum; common	Associated with NGU, vaginitis, septic abortion, infertility, congenital pneumonia

Table adapted from Krause and Taylor-Robinson (1992).

1. 3. 2. 1. M. PNEUMONIAE

Mycoplasma pneumoniae is the major cause of primary atypical pneumonia in humans and causes a febrile bronchitis or pneumonia often accompanied with generalised symptoms such as malaise, muscular pain, sore throat or headache. The organism may be carried in the throat for several months after natural clinical recovery. Infection is probably worldwide although most reports are from northern temperate zones and Australasia. The mycoplasma attaches to a receptor(s) on the cilia of cells of the respiratory epithelium by means of binding proteins that are located on a specialised terminal structure. The correct formation of this tip structure is a prerequisite for a successful infection (Krause, 1998). A 165kd protein, designated P1, is involved in this cytadherence process (Kahane *et al.*, 1985) but other proteins are also required (Baseman, 2000). These include P30, and two further membrane proteins of 40 and 90 kDa (Droesse *et al.*, 2000), and a number of high molecular weight proteins that form part of the cytoskeleton-like structure (Krause, 1998). These proteins are proline-rich and exhibit repeat sequences and other motifs characteristic of eukaryotic cytoskeletal proteins (Hahn *et al.*, 1998).

1. 3. 2. 2. M. HOMINIS

Mycoplasma hominis is frequently found as part of the normal flora in the human urogenital tract. (Krause and Taylor-Robinson, 1992). However, it is associated with acute upper urinary tract infections, which are often accompanied by the development of a significant specific antibody response (Thomsen, 1978). It is also associated with pelvic inflammatory disease, which is a cause of infertility in women. This disease usually results from infection by *Chlamydia trachomatis* or *Neisseria gonorrhoeae*; however *M. hominis* is often isolated from patients and may have a synergistic role with these pathogens in causing disease. A review of *M. hominis* in extra-urogenital tract disease (Madoff and Hooper, 1990) identified five additional categories of infection: septicaemia, joint infection, central nervous system infection, respiratory infection, and wound infection, particularly after surgery. *M. hominis* has also been implicated in chorioamnionitis, and respiratory distress in the new-born (Krause and Taylor-Robinson, 1992). In the

immunocomprimised host *M. hominis* can cause significant morbidity and mortality (Meyer and Clough, 1993), which is compounded by the organism's resistance to erythromycin and the newer macrolides (Gass *et al.*, 1996). It can be a significant problem in organ transplantation and has been reported to be transmitted from donor to recipient in lung allografts (Gass *et al.*, 1996) and bone marrow (Kane e*t al.*, 1994), liver (Haller *et al.*, 1993), renal (Miranda *et al.*, 1990) and heart transplants (Boyle *et al.*, 1993).

1. 3. 2. 3. M. FERMENTANS

Mycoplasma fermentans was first isolated from a human genital ulcer (Ruiter and Wentholt, 1952) and has since been isolated most frequently from the urogenital tract. It has also been isolated from joint fluids and may have a role in rheumatoid arthritis (Somerson and Cole, 1979). More recently *M. fermentans* has been linked with autoimmune deficiency syndrome (AIDS)-associated nephropathy and systemic infections in AIDS patients (Lo *et al.*, 1989). Furthermore, it has been shown to be a cofactor in causing fatal systemic infections in HIV-negative individuals (Lo *et al.*, 1989). It is also a known contaminant of cell cultures and has been recently isolated from sheep (Nicholas *et al.*, 1998).

1. 3. 2. 4. M. GENITALIUM

Humans are the natural host for *M. genitalium*, which has the smallest genome of any free-living organism (Colman *et al.*, 1990; Table 1. 2.). However, very few isolations have been reported and its primary localisation within the human host is still a subject for speculation (Bott and Fraser, 1998). The presence of the organism has been detected by PCR in arthritic joints, the blood of AIDS patients, the respiratory tract of an immunodeficient patient and in urethral swabs. It has a high level of association with non-gonococcal urethritis and pelvic inflammatory disease and may be a factor in the aetiology of the disease. Serological detection of *M. genitalium* infection has been hampered by its cross-reactivity with *M. pneumoniae* (Tully *et al.*, 1995). These organisms are morphologically similar both possessing a specialised terminal organelle which plays a role in attachment to host cells and is a prerequisite for infection (Herrmann and Reiner, 1998).

1. 3. 2. 5. UREAPLASMA UREALYTICUM

Ureaplasma urealyticum can be divided into two biotypes, based on genome size and rDNA sequence, and 14 serotypes (Pitcher *et al.*, 2000). It is a common inhabitant of the urogenital tract and has been associated with non-gonococcal urethritis. In addition, compared to control groups, ureaplasma-positive placentas are observed more frequently from women with chorioamnionitis, premature birth, fetal loss, premature rupture of fetal membranes and low-weight new borns (Cunha, *et al.*, 1997). Chang *et al.*, (2000) also detected *U. urealyticum* DNA in cervical and ovarian cancer tissues and speculated that the organism may be a co-factor in cancer development. Ureaplasmas are also found in many animal species.

1. 3. 2. 6. AIDS ASSOCIATED MYCOPLASMAS

Infectious agents other than HIV-1 appear to play an important role in promoting the disease progression of AIDS. Systemic mycoplasmal infections may explain a number of unusual disease processes, including multiple organ failure and autoimmune disorders (Lo, 1992). The mycoplasmas that have been linked with AIDS are *M. fermentans*, *M. pirum*, *M. genitalium* and *M. penetrans* (Lo, 1992). Interestingly, all of these mycoplasmas are glucose fermenters and only one of them, *M. genitalium* does not additionally hydrolyse arginine. *M. fermentans* (incognitus strain) was isolated during transfection studies of cells with DNA extracted from Kaposi's sarcoma tissue derived from an AIDS patient. This strain was shown to enhance the cytotoxic effect of HIV-I on CD4 lymphocytes in culture (Lo *et al.*, 1991). However, the unusual manner in which *M. fermentans* incognitus was isolated has been viewed with some scepticism; there is no precedent for nucleic acid from a prokaryotic organism being infective for eukaryotic cells (Van der Riet, 1990). It is more likely that either the transfected DNA or the tissue culture cells were contaminated with *M. fermentans* (Hannan, 1997). Further work is necessary to establish the role of mycoplasmas in AIDS.

1. 3. 3. MOLLICUTES IN ANIMALS

Since *M. mycoides* was first described in 1898 mollicutes have been isolated from almost every animal species that has been studied. Infections are often associated with diseases of the lung, urogenital tract and joints. Several diseases

are of economic importance in agriculture and some affect biomedical research by infecting animals used in experimental models. This section on mollicutes in animals will focus on major disease causing species.

1. 3. 3. 1. MOLLICUTES OF LABORATORY RODENTS

Experimental animals need to be free of indigenous pathogens and subclinical infections to ensure accurate and reproducible research data. Mycoplasmal diseases in rats and mice are a recognised problem (Cassell and Hill, 1979) and there is substantial evidence that infections with *M. pulmonis* and *M. arthritidis* have interfered with a number of studies (Lindsey *et al.*, 1986).

Mycoplasma pulmonis is the cause of murine respiratory mycoplasmosis and nearly all rat and mice colonies in the United States are infected (Ferebee *et al.*, 1992). Generally the disease is asymptomatic, but rhinitis, laryngitis, tracheitis, bronchitis, bronchiectasis and alveolitis occur, often in association with other pathogens (van Kuppeveld *et al.*, 1993). Rabbits, hamsters and guinea pigs may also be affected. The mycoplasmas that have been isolated from rats and mice are listed in Table 1. 5. (van Kuppeveld *et al.*, 1993).

TABLE 1. 5. *MOLLICUTES* ISOLATED FROM RATS AND MICE

MOLLICUTE SPECIES	NATURAL HOST(S)	FREQUENCY OF INFECTION	NATURAL DISEASE
Mycoplasma arthritidis	Rats, mice	Common	Arthritis
Mycoplasma collis	Rats, mice	Unknown	Unknown
Mycoplasma KE2	Rats	Unknown	Unknown
Mycoplasma muris	Mice	Unknown	Unknown
Mycoplasma neurolyticum	Mice	Rare	Not reported but able to cause experimental infection
Mycoplasma pulmonis	Rats, mice	Common	Infection of respiratory tract, genital disease and arthritis.

Table compiled from data in van Kuppeveld *et al.* (1993) and Simecka *et al.* (1992).

1. 3. 3. 2. MOLLICUTES OF POULTRY AND BIRDS

Currently, 20 mollicute species have been isolated from poultry and birds. The most economically important are *M. gallisepticum, M. synoviae, M. meleagridis* and *M. iowae* (Fan *et al.*, 1995). All of these pathogens are egg-transmitted. *M. gallisepticum* causes chronic respiratory disease in chickens and infectious sinusitis in turkeys (Simecka *et al.*, 1992). Luttrell *et al.* (1996) also isolated *M. gallisepticum* from house finches affected with conjunctivitis. *M. synoviae* is recognised as a pathogen of both chickens and turkeys causing severe respiratory disease and synovitis (Kleven *et al.*, 1991). *M. meleagridis* occurs in turkeys, Japanese quail and peacocks. It is considered a true venereal disease,

associated only rarely with respiratory disease in adults, but causing air sacculitis in embryos and young birds (Simecka *et al.*, 1992). *M. iowae* is associated with reduced hatchability of turkey eggs, and possibly, immunosuppression in turkey poults. It has been shown to cause mortality of both turkey and chicken embryos as well as mild to moderate air sacculitis and leg abnormalities in experimentally inoculated chickens and turkeys (Abdul-Rahman *et al.*, 1996). Other mycoplasmas of possible economic significance in poultry disease are *M. anatis*, a cause of air sacculitis in ducklings, and *M. gallinarum*. This has been reported as a potential pathogen in chickens, where it appears as a secondary invader following certain viral infections (Shah-Majid, 1996).

A number of *Mycoplasma* species have been isolated from wild birds. Forsyth *et al.* (1996) named *M. sturni* from a starling, and Poveda *et al.* (1994) *M. buteonis, M. falconis,* and *M. gypis* from birds of prey.

1. 3. 3. 3. MOLLICUTES OF SWINE

Many mollicutes have been isolated from pigs, but the main swine disease causing mycoplasmas are *M. hyopneumoniae, M. hyorhinis* and *M. hyosynoviae. M. hyopneumoniae* is the causal agent of enzootic pneumonia of swine, a chronic respiratory disease that occurs worldwide (Simecka *et al.*, 1992). This mycoplasma is very difficult to grow on artificial media, and was not isolated until 1965 (Goodwin *et al.*, 1967). Both *M. hyorhinis* and *M. hyosynoviae* are usually found in the upper respiratory tract of adult pigs, where they act as reservoirs of infection, causing severe polyarthritis in pigs aged 3 to 6 months (Simecka *et al.*, 1992).

1. 3. 3. 4. MOLLICUTES OF SHEEP AND GOATS

Mycoplasma infections in sheep and goats often result in pneumonic disease, however arthritis and septicaemia are also common. Some mycoplasma infections are specifically associated with mastitis or keratoconjunctivitis. One of the most important pneumonic diseases is contagious caprine pleuropneumonia (CCPP) caused by *M. capricolum* subsp. *capripneumoniae*, formerly known as *Mycoplasma* F38 (Bonnet *et al.*, 1993). This is characterised by red and grey hepatization of the lung with characteristic haemorrhagic infarction that may involve

an entire lobe. Previously, CCPP had been linked to other mycoplasmas including *M. capricolum* subsp. *capricolum, M. mycoides* subsp. *capri* and *M. mycoides* subsp. *mycoides* LC (large colony), which cause a disease similar to CCPP. However, MacOwan (1984) showed that only *M. capricolum* subsp. *capripneumoniae* fulfilled Koch's postulates in causing the classical signs of disease, which include a characteristic highly infectious pulmonary pneumonia. Recently, it has been concluded that *M. mycoides* subsp. *capri* and *M. mycoides* subsp. *mycoides* (large colony) should be classified within the same taxon (Anon, 2000). This is based on genetic, biochemical and serological evidence although DNA homology studies (Table 1. 8; Section 1.5.3.) are able to separate the two groups.

Mycoplasma mycoides subsp. *mycoides* SC (small colony) has also been isolated from sheep and goats (Cottew and Yeats, 1978; Brandão, 1995) and has recently been associated with contagious caprine pneumonia in India and Eastern Tanzania (Kusiluka *et al.*, 2000; Srivastava *et al.*, 2000). This organism is the cause of contagious bovine pleuropneumonia (CBPP) in cattle and its isolation from other animals has implications for disease control (see Section 1.5).

Contagious agalactia (CA) is an important disease of sheep and goats that occurs worldwide, particularly where herds are intensively reared. *M. agalactiae* is the classical agent of CA, but *M. capricolum* subsp. *capricolum*, *M. mycoides* subsp. *mycoides* (large colony) and *M. putrefaciens* also contribute to this syndrome. The disease is predominantly chronic and causes agalactia, mastitis, keratoconjunctivitis, arthritis and septicaemia, which may result in mortality. The financial loss is considerable with one recent estimate for Greece alone of approximately 24.5 million ECU per annum (Legakis, 1995).

Mycoplasma ovipneumoniae is found as a commensal organism of the respiratory and genital tracts of sheep and goats, but can produce a primary pneumonic infection that may be complicated by secondary invaders such as *Mannheimia* (*Pasteurella*) *haemolytica* (Jones *et al.*, 1982).

Infectious keratoconjunctivitis ('pinkeye') of sheep and goats is caused by *M. conjunctivae.* Once introduced into an immunologically naive herd, *M. conjunctivae* can spread rapidly, but infection tends to be self-limiting and permanent lesions are rare (Giacometti *et al.*, 1996; Naglić *et al.*, 2000).

Mycoplasma arginini and *M. bovigenitalium* have been isolated from the genital tracts of sheep in Nigeria (Chima *et al.*, 1995) and experimental infections with *M. bovigenitalium* in sheep have caused vulvovaginitis; however the role of mycoplasmas in naturally-acquired genital disease is not clear. The recent isolation of *M. fermentans* from a case of vulvovaginitis in a sheep is surprising (Nicholas *et al.*, 1998). VLA Weybridge has now received three separate isolates of *M. fermentans* from sheep, all associated with genital tract infections (Nicholas, R. A. J., Ayling, R. D., unpublished observations).

1. 3. 3. 5. MOLLICUTES OF CATTLE

Mollicutes isolated from cattle are listed in Table 1. 6. *M. mycoides* subsp. *mycoides* SC the causative agent of CBPP, and *M. bovis*, a cause of bovine mastitis and respiratory infection in calves, are the major organisms of study in this thesis and are considered separately in Sections 1.4 and 1.5 respectively. The significance of other species is discussed below.

Mycoplasma alkalescens has been isolated from the synovial fluid of calves and has been suggested to be a rare cause of arthritis (Whithear, 1983). It was isolated from 8.5% of 658 cattle with respiratory disease in Northern Italy during 1994 to 1996 (Manfrin *et al.*, 1998). Since nearly 80% of the tested animals were negative for mycoplasmas, *M. alkalescens* would appear to be an important member of the mycoplasma flora of cattle and might potentially be important in disease (Reeve-Johnson, 1999).

Mycoplasma bovigenitalium appears to be ubiquitous having been reported throughout the world (Reeve-Johnson, 1999). It is associated with respiratory disease and has been isolated from the lower respiratory tract. It has also been isolated from mastitic milk and the genital tract and prepuce of bulls with symptoms of infertility (Atef and Mohy, 2000). Interestingly, recent work on UK sheep isolates, identified as Mycoplasma ovine serogroup 11, has shown that this group is not distinct from *M. bovigenitalium* and should be considered as part of the same species (Nicholas *et al.*, in press).

Mycoplasma bovirhinis is frequently isolated from cattle in the UK and has been shown to cause mastitis (Simecka *et al.*, 1992). It has also been isolated from the respiratory tract, joints and eyes (Thomas and Smith, 1972; Reeve-Johnson,

1999), but is not considered to be pathogenic at these sites; possibly it outgrows more fastidious pathogenic mycoplasmas in isolation media.

Mycoplasma canadense and *M. californicum* are known to cause mastitis in cattle (Simecka *et al.*, 1992). *M. canadense* was first isolated in Canada from cases of mastitis, swollen joints and umbilicus of a calf, vaginal discharge of a cow and semen of a bull (Ruhnke and Onoviron, 1975). Ball *et al.* (2000) investigated an outbreak of clinical mastitis in newly-born calves in Northern Ireland and isolated *M. canadense*; however antigen to *M. californicum* was also detected in serum samples using a sandwich ELISA. Kaur *et al.* (1998) recently reported in India, the first isolation of *M. canadense* from the milk of mastitic cows and buffaloes.

Mycoplasma dispar can induce pneumonia (Ross, 1993), although it has also been isolated from healthy cattle. Diagnosis is difficult, as the disease is often subclinical and the organism takes up to 14 days to grow, often being 'outgrown' by more prolific but less important mycoplasmas. This poor growth rate may account for its apparent restricted geographic distribution, with isolations only being made in Western European countries, the USA and Australia (Reeve-Johnson, 1999). The use of an antigen-capture ELISA on bronchoalveolar lavage fluids from Belgium cattle suggested that *M. dispar* was more common than *M. bovis* (Thomas *et al.*, 2000).

Mycoplasma sp. bovine serogroup 7 is one of six recognised members of the *M. mycoides* cluster (See Table 1.8, Section 1.5.3.). It was first isolated from arthritic calves in South Queensland, Australia in the early 1960's. It has a history of causing sporadic outbreaks of arthritis in calves and mastitis in cows but has also been recovered from pneumonic bovine lungs and lymph nodes (Cottew, 1970). In a recent severe outbreak of polyarthritis, mastitis and abortion in three large dairies in New South Wales, Australia, bovine serogroup 7 was the only pathogenic agent recovered (Djordjevic *et al.*, 2000).

Mycoplasma bovoculi is associated with keratoconjunctivitis in cattle (Simecka *et al.*, 1992). *M. canis* is normally associated with dogs but is increasingly being isolated from pneumonic cattle (Nicholas *et al.*, 1995; 2000b) and recently has been identified in bovine mastitic milk samples (Ayling R., unpublished data). Other *Mycoplasma* species isolated from cattle include *M. arginini, M. alvi* and *M.*

verecundum. However, although these organisms may be isolated from diseased cattle, it is unclear whether they cause disease.

Acholeplasma laidlawii is frequently isolated from cattle and its rapid growth in media commonly used for isolating mycoplasmas may obscure other more important organisms. Its role as a pathogen has not been shown.

Ureaplasma species are known to cause calf pneumonia, genital disease and conjunctivitis (Simecka *et al.,* 1992). *Ureaplasma diversum* can be isolated from both acute and chronically diseased respiratory tracts, and is capable of producing mild sub-clinical pneumonia. It is possible that its main role is in rendering the respiratory tract more susceptible to complicating bacterial infections (Reeve-Johnson, 1999).

TABLE 1. 6. *MOLLICUTES* THAT AFFECT CATTLE

MOLLICUTE SPECIES	DISEASE
Mycoplasma mycoides subsp.	Contagious bovine pleuropneumonia (CBPP).
mycoides SC	See Section 1. 5.
Mycoplasma bovine	Calf pneumonia and arthritis
serogroup 7	
Mycoplasma arginini	Not reported to be pathogenic
Mycoplasma alkalescens	Arthritis
Mycoplasma bovigenitalium	Mastitis and genital disease
Mycoplasma bovirhinis	Mastitis
Mycoplasma bovis	Calf pneumonia, mastitis and arthritis, See Section 1. 4.
Mycoplasma bovoculi	Conjunctivitis
Mycoplasma canis	Calf pneumonia?, mastitis?
Mycoplasma californicum	Mastitis
Mycoplasma canadense	Mastitis
Mycoplasma dispar	Calf pneumonia and mastitis
Mycoplasma verecundum	Not reported to be pathogenic
Acholeplasma spp	Not reported to be pathogenic
Ureaplasma spp.	Calf pneumonia, genital disease, and conjunctivitis

Table adapted from Simecka *et al.* (1992).

1. 3. 3. 6. MOLLICUTES OF OTHER ANIMALS

A number of mycoplasma species have been isolated from horses. Their true pathogenic significance is unknown, but there is evidence that *M. felis* causes pleuritis (Wood *et al.*, 1997) and this mycoplasma has been associated with feline conjunctivitis and pneumonia. *M. gateae* and *M. feliminutum* have also been implicated in feline pneumonia. Using 16S rRNA gene sequencing and serological methods, a mycoplasma isolate from a seal was recently (October 2000) identified at

VLA (Weybridge) as *M. phocacerebrale.* In dogs, *M. cynos* causes a rapidly spreading respiratory infection involving the lungs and *M. canis* and *M. spumans* are reported to cause urinary tract infections. Recent reports by Giacometti *et al.* (1996) have described outbreaks in Switzerland of conjunctivitis in ibex, chamois and sheep caused by *M. conjunctivae.* Apparently, affected wild animals are unable to see and as a consequence they fall off the mountains.

Recent developments in mycoplasmology, especially genetic typing methods, have also led to the description of new species from more unusual animals, such as: elephants (*Mycoplasma elephantis* sp. nov., Kirchhoff *et al.,* 1996); tortoises (*M. agassizii* sp. nov., Brown *et al.*, 1995); crocodiles (*M. crocodyli* sp. nov., Kirchhoff *et al.*, 1997); and Afghan pikas (a type of small rabbit) (*M. lagogenitalium* sp. nov., Kobayashi *et al.,* 1997). Other examples of mollicutes found in various animal species are given in Table 1. 7.

TABLE 1. 7. EXAMPLES OF *MOLLICUTES* FOUND IN DIVERSE ANIMAL SPECIES

ANIMAL SPECIES	MOLLICUTES SPECIES
Camel	*Acholeplasma oculi*
Fish (Tench)	*Mycoplasma mobile*
Giraffe	*Acholeplasma laidlawii*
Squirrel	*Mycoplasma citelli*
Hedgehog	*Acholeplasma laidlawii*
Lion	*Mycoplasma leocaptivus*
	Mycoplasma leopharyngis
	Mycoplasma simbae
Llama	*Mycoplasma bovis*
Mouflon (wild sheep)	*Acholeplasma laidlawii*
Puma	*Mycoplasma felifaucium*
Seal	*Mycoplasma phocacerebrale*
	Mycoplasma phocarhinis
	Mycoplasma phocidae

Table adapted from Rosenbusch. (1994).

In addition, although mycoplasmas are usually host specific, the list of mollicute species isolated from nontypical hosts is increasing with reports of: *M. canis* in cattle (Nicholas *et al.,* 1995); *M. salivarum,* a common human buccal mycoplasma, in pigs; *Acholeplasma oculi,* a goat mycoplasma in human amniotic fluid (Razin, 1992); and *M. fermentans* in sheep (Nicholas *et al.,* 1998). Whether these mycoplasmas cause disease, are part of the normal flora, or are opportunistic pathogens is unknown.

1. 4. MYCOPLASMA BOVIS

Mycoplasma bovis has been associated with many bovine diseases, including pneumonia, arthritis, mastitis, subcutaneous abscesses, keratoconjunctivitis, meningitis, infertility (Behrens *et al.*, 1996; Kirby and Nicholas, 1996), otitis media (Walz *et al.*, 1997) and abortion (Byrne *et al.*, 1999). It was first isolated in 1961 from severe bovine mastitis in the USA and initially called *M. agalactiae* var (later subsp.) *bovis* because of similarities, both clinical and biochemical, to *M. agalactiae*. *Mycoplasma bovis* is the most frequently occurring pathogenic bovine mycoplasma in Europe and America (Nicholas, 1997) and the second most important mycoplasma pathogen affecting cattle (Ter Laak *et al.*, 1992). In a survey by the OIE covering over 48 countries, *M. bovis* was seen as a major cause of loss in the cattle industry. In a US survey, *M. bovis* was isolated from one third of over 400 pneumonic lungs. In France, *M. bovis* is involved in 25-30% of pulmonary disease in fattening calves (Nicholas *et al.*, 1999). In Britain, *M. bovis* is the most common mycoplasma pathogen of cattle and is estimated to cause losses of as much as £50 million per year in treatment, 'set back' costs and deaths in calves (Rebhun *et al.*, 1995). The first UK isolation of *M. bovis* was from a severe case of calf pneumonia in Southern England in 1974 (Thomas *et al.*, 1975). The organism has also been associated with severe arthritis, synovitis, genital tract lesions, and mastitis in UK herds and in 1995 about 25 % of all fatal cases of calf pneumonia in N. Ireland were associated with *M. bovis* infection (Ball *et al.*, 1995).

Mycoplasma bovis may act synergistically in disease with other organisms such as *Pasteurella* species, *Haemophilus somnus* and *Actinomyces pyogenes* (Doherty *et al.*, 1994; Rebhun *et al.*, 1995). However, it is increasingly being considered as a primary pathogen. It has been shown that *M. bovis* can induce pneumonia and arthritis in gnotobiotic calves (Gourlay *et al.*, 1976) and in 'normal' calves (Stipkovits *et al.*, 2000b). Infected calves start to develop clinical signs within eight to ten days of challenge. Clinical signs include depression, hyperpnea, dyspnea, respiratory distress, various degrees of nasal discharge, coughing, fever and loss of appetite. Body weight is also significantly lower than in uninfected animals. The calves stand with head and neck extended and forelegs spread apart. Pneumonia caused solely by *M. bovis* is generally referred to as a 'cuffing

pneumonia' because lymphoid hyperplasia appears around the airways. Lesions are usually limited to the cranial tips of the lung lobes and there may be a purulent bronchiolitis (Pfutzner and Sachse, 1996).

In experimental infections calves may die within 3 weeks of challenge (Stipkovits *et al.*, 2000b). Stipkovits *et al.*, (2000b) described the development of pneumonia characterised by either: perivascular and peribronchial infiltration of lymphoid cells; catarrhal pneumonia with desquamation of epithelial cells and accumulation of serous exudate in alveoli and bronchi; or the presence of inflamed foci surrounded by connective tissue. He also recovered *M. bovis* from the lung, spleen, livers, kidneys and joints. Rodriguez *et al.* (1996*c*) described similar findings in experimentally infected calves; there was consolidation of lungs, although they observed little or no desquamation of epithelial cells. Immunohistochemical findings revealed large quantities of *M. bovis* antigen, particularly at the edges of necrotic zones in association with pyknotic cells, and also in epithelial cells, within inflammatory cells in airway lumina, and in alveolar walls (Rodriguez *et al.*, 1996b).

Mycoplasma bovis infection is invariably brought onto a farm by clinically normal cattle that shed the organism in exhalation droplets. Subsequent transmission of *M. bovis* infection then occurs *via* the respiratory tract, by contact with contaminated milking equipment, or by calves suckling infected cows (Pfutzner and Sachse, 1996).

Control of the disease is difficult. Currently no vaccines exist and antimicrobial therapy is of limited use. Specific antimicrobial treatment for mycoplasmas is usually considered only when treatments with antimicrobials effective against other pneumonia causing bacteria, such as *Pasteurella* and *Haemophilus*, have been unsuccessful. The most widely used antimicrobials against these cell-walled bacteria are penicillin and ampicillin which have modes of action that will not affect mycoplasmas. Thus by the time appropriate antimicrobials, such as tilmicosin, spectinomycin or the fluoroquinolones, are administered, *M. bovis* infection is advanced.

An immune response is mounted against *M. bovis* infection. However, it does not usually lead to high antibody titres, although titres depend on the severity

of infection and the diagnostic test used (Nicholas, 1997). Feenestra *et al.* (1991) investigated the diagnosis of *M. bovis* mastitis using an indirect haemagglutination test (IHA) and found titres varying between less than 1/160 to greater than 1/1280 in affected calves. Most of the calves were culled soon after the infection was detected, but one cow was still shedding *M. bovis* 10 months later.

Nagatomo *et al.* (1996) investigated the immune response using the IHA test for three groups of calves introduced to a farm with a history of *M. bovis* infection. One group was treated with the antimicrobial leucomycin as a preventative therapy. The untreated groups showed significant antibody titres and clinical signs of disease after 60 days. In contrast, in the leucomycin-treated group, antibody response and clinical signs were not noted until after 248 days. An indirect ELISA (Nicholas *et al.*, 2000b) for *M. bovis* has been in use at VLA (Weybridge) for several years to screen cattle in Britain. This has given positive results for approximately 20-25% of serum samples submitted from pneumonic calves. Byrne *et al.* (2000) successfully used the same ELISA to identify antibodies in milk samples collected from a herd with *M. bovis* mastitis.

Culturing from the affected animal is the usual method of diagnosis for *M. bovis* infection. *M. bovis* is one of the more rapidly growing mycoplasmas. It characteristically does not ferment glucose, but oxidises lactate or pyruvate (Miles, 1992) and produces a film on culture media (Poveda and Nicholas, 1998). Identification of isolates is usually achieved using specific polyclonal antisera in a disc film inhibition test or in an indirect fluorescent antibody test (Bradbury, 1998a). The use of PCR technique may also improve *M. bovis* detection (see chapter 2). However, several workers are currently investigating selective, chromogenic isolation media that will enable the rapid and direct detection of *M. bovis* by their colony colour on agar plates. (Khan *et al.*, 2000; Windsor and Bashiruddin, 2000).

One possible problem in the identification of *M. bovis* isolates by conventional serological methods, is surface antigenic variation. The prominent membrane surface lipoproteins (vsps) of *M. bovis* are immunogenic and undergo dynamic and spontaneous changes in size and expression (Behrens *et al.*, 1996). Antigen size variation occurs through the gain or loss of tandem repeated units in the *vsp* gene. Oscillation in vsp expression may be due to DNA inversion or DNA

insertion and is currently being investigated (Citti *et al.*, 1999). Sachse *et al.* (1999) has shown that vsp antigens are important in cytoadhesion.

1. 5. CONTAGIOUS BOVINE PLEUROPNEUMONIA (CBPP)

1. 5. 1. THE CLINICAL DISEASE

Contagious bovine pleuropneumonia (CBPP) is included in List A of the Office International des Epizooties (OIE, 1996). It is among the six most important diseases under the FAO Emergency Prevention System (EMPRES) (Rweyemamu and Benkirane, 1996) along with diseases such as rinderpest and foot and mouth disease. It predominantly affects cattle, but may additionally cause disease in water buffalo, yak, reindeer and bison (Provost *et al.*, 1987); the causative organism *Mycoplasma mycoides* subsp. *mycoides* SC has also been isolated from sheep and goats (Cottew and Yeats, 1978; Brandão, 1995). Transmission of the organism is primarily by droplet infection (aerosols), fine bronchial secretions being expelled over short distances when diseased animals cough. In spite of this airborne mode of transmission, CBPP spreads slowly amongst herds but once established the disease persists in an endemic form.

The range of clinical signs seen in cattle affected by CBPP vary from hyperacute through acute to chronic and sub-clinical forms. Respiratory distress and coughing, evident on physical stimulation of resting animals, are the main signs (Scudamore, 1995). The incubation period of the natural disease may range from 5 to >200 days but most disease occurs within 40 days (Egwu *et al.*, 1996).

The earliest sign of infection in dairy cattle may be a fall in milk production. In the acute form, the early stages of disease are indistinguishable from any severe pneumónia with pleurisy (Scudamore, 1995). Animals show dullness, anorexia, and irregular rumination with moderate fever and may show signs of respiratory distress. Coughing is usually persistent and is dry. Sometimes body temperature rises from 40 to 42°C and the animal prostrates with difficulty of movement. As the lung lesions develop, the symptoms become more pronounced with increased frequency of coughing and the animal may become prostrate or stand with back arched, head extended and foreleg knee joints abducted. In calves up to six months old, CBPP may manifest itself as arthritis, and the chest may be free from lesions. A small number of affected calves die after mild exertion due to valvulitis, and on rare occasions myocarditis (Martel *et al.*, 1983).

Clinical signs are accelerated in the hyperacute form of the disease and affected animals may die within a week of exhibiting classical respiratory symptoms. In the sub-acute form, symptoms may be limited to a slight cough, only noticeable when the animal is exercised.

In Africa mortality rates are typically between 10-70% and are particularly high in newly affected regions; however, in parts of Europe where the disease is endemic, there is no mortality and the majority of infected cattle show chronic lesions (Egwu *et al.*, 1996).

Nicholas and Palmer (1994) suggested that this difference might be due to European stock being housed permanently, less stressed and healthier than those in Africa, which often have a nomadic existence. The wider use of antimicrobials in Europe may also mask the clinical signs and accelerate the formation of chronic lesions in carrier animals (Provost *et al.*, 1987). However, there are genetic and biochemical differences between European and African strains of *M. mycoides* subsp. *mycoides* SC, which may suggest differences in virulence (see Section 1.5.3).

1. 5. 2. PATHOLOGY

The pathological lesions of the disease are generally confined to the thoracic cavity and lungs. The lesions are usually unilateral, showing no preference for the right or left lung although the diaphragmatic lobe is more commonly affected than the cranial lobe (Nunes Petisca *et al.*, 1990). The thoracic cavity of affected animals may contain many litres of clear yellowish brown fluid containing some pieces of fibrin (Ter Laak, 1992). Caseous fibrinous deposits are observed on the parietal and visceral surfaces of the lungs (Provost *et al.*, 1987). Other pneumonia causing organisms such as *Mannheima haemolytica* usually affect both lungs.

The interlobular septa of the affected lung show distension with amber-coloured fluid surrounding the distended lymphatics. This fluid separates the lung lobules which vary in colour with red, grey and yellow hepatisation being evident and representing different stages of inflammatory lesions (Hudson, 1971). Consolidation of the lungs with typical marbled appearance, sometimes accompanied by adhesion of the parietal and visceral surfaces, is also characteristic. In chronic or advanced cases, a sequestrum of variable size, ranging from a few mm to 20 cm, and consisting of necrotic lung parenchyma surrounded by a fibrous

capsule, is formed (Martel *et al.,* 1983; Trichard *et al.,* 1989; Santini *et al.,* 1992; Nicholas *et al.,* 1996; see Chapter 4, figure 4.3).

An immunocytochemical study of CBPP affected lungs by Scanziani *et al.* (1991) showed that the severity of lung lesions correlated with the severity of changes in the lymph nodes. In the acute stage of the disease, specific antigen was detected in the lobular periphery and in the cytoplasm of alveolar macrophages. Chronic lesions showed immunoreactivity in the fibrotic areas and in the macrophages located in the lobular septa. Necrotic debris and macrophages located in the inner part of the sequestra were specifically stained. Immunoreactive material was also seen in the centrofollicular areas of the broncho-associated lymphoid tissue structures and in the lymph follicles of fibrotic septa (Bashiruddin *et al.,* 1999). Electron microscopy of the mediastinal lymph nodes of a chronically affected calf also showed intact and degenerating mycoplasmas within macrophages (Bashiruddin *et al.,* 1999).

In acute cases, lesions have also been observed in the kidneys (Hudson, 1971), and calcifications as a result of CBPP are reported as distinctive and easily differentiated from the common forms of bovine interstitial nephritis (Scanziani *et al.,* 1999). *M. mycoides* subsp. *mycoides* SC has been isolated from the kidneys and urine of cattle affected with CBPP (Scudamore 1976). An immunohistochemical study (Scanziani *et al.,* 1999) demonstrated the presence of *M. mycoides* subsp. *mycoides* SC antigen in the kidneys of acute and chronically affected animals, with antigen detected in several renal structures including the lumen of renal vessels, glomeruli, interstitial cells and tubular epithelia. Scanziani *et al.* (1997) demonstrated that *M. mycoides* subsp. *mycoides* SC antigen could often be detected immunohistochemically in cases where mycoplasma culture had yielded negative results. This may suggest the presence of detectable antigen after the loss of mycoplasma viability.

1. 5. 3. THE CAUSATIVE ORGANISM

The causative agent of CBPP, *M. mycoides* subsp. *mycoides* SC, is one of six closely related mycoplasmas referred to as the *M. mycoides* cluster. The other members of the cluster are: *M. mycoides* subsp. *mycoides* LC, *M. mycoides* subsp. *capri, M. capricolum* subsp. *capricolum, M. capricolum* subsp. *capripneumoniae,*

and *Mycoplasma* bovine serogroup 7. All members of the cluster are pathogens of ruminants. *M. capricolum* subsp. *capripneumoniae* (see Section 1.3.3.4) is the causative agent of contagious caprine pleuropneumoniae (CCPP), an OIE list B disease. CCPP is highly contagious and occurs in Africa and the Middle East, causing fever, coughing, painful respiration, depression and up to 70% mortality (Rweyemamu and Benkirane, 1996). Typical CCPP pathology shows fibrinous pleuropneumonia and intralobular oedema in lungs.

Mycoplasma mycoides subsp. *mycoides* LC, *M. mycoides* subsp. *capri*, *M. capricolum* subsp. *capricolum* and *M. putrefaciens* (see Section 1.3.3.4) are all associated with sheep and/or goats, causing mastitis, arthritis and keratoconjunctivitis; *M. capricolum* subsp. *capricolum* is also associated with ulcerative vulvovaginitis in sheep (Sarris, 1996).

The relationship between the members of the *M. mycoides* cluster, based on DNA-DNA hybridisation, is shown in Table 1. 8. From this table it appears that *M. mycoides* subsp. *mycoides* LC and SC are the most closely related, with 82% homology, whilst the two *M. capricolum* subspecies also have a high (80%) homology. However, based on sequence analysis, a different pattern of relationships has emerged. All members of the *M. mycoides* cluster have two rRNA operons, *rrnA* and *rrnB* and Johansson *et al.* (1996a) sequenced both 16S rRNA genes. This revealed two subclusters: *M. mycoides* subsp. *capri* and *M. mycoides* subsp. *mycoides* LC, the *M. capri* subcluster; and *M. capricolum* subsp. *capricolum, M. capricolum* subsp. *capripneumoniae* and *Mycoplasma* bovine serogroup 7, the *M. capricolum* subcluster. Interestingly, *M. mycoides* subsp. *mycoides* SC was grouped in the *M. capri* subcluster using *rrnA* sequences and the *M. capricolum* subcluster with the *rrnB* sequences. Based on 16S rRNA analysis Pettersson *et al.* (1996) suggested that *M. mycoides* subsp. *mycoides* LC and *M. mycoides* subsp. *capri* should form a single taxon; this is also supported by biochemical and serological evidence. Sequence analysis of 16S rRNA genes has also shown that *M. putrefaciens* is closely related to members of the *M. mycoides* cluster (Johansson *et al.*, 1996a).

TABLE 1. 8. HOMOLOGY OF STRAINS WITHIN THE *M. MYCOIDES* CLUSTER BASED ON DNA-DNA HYBRIDISATION

	DNA-DNA HYBRIDISATION (% of homologous hybridisation)					
ORGANISM	PG1	Y-GOAT	PG3	California kid	PG50	F38
Mycoplasma mycoides subsp. *mycoides* SC PG1	100					
Mycoplasma mycoides subsp. *mycoides* LC Y Goat	82	100				
Mycoplasma mycoides subsp. *capri* PG3	70	75	100			
Mycoplasma capricolum subsp. *capricolum* California kid	ND	41	40	100		
Bovine serogroup 7 PG50	ND	72	57	55	100	58
Mycoplasma capricolum subsp. *capripneumoniae* F38	ND	36	39	80	60	100

ND, not determined

Table adapted from Christiansen (1992).

Amongst *M. mycoides* subsp. *mycoides* SC strains, there appears to be marked differences between isolates from Europe and those from Africa and Australia. Increased mortality in African disease outbreaks compared to European outbreaks has already been mentioned in section 1.5.1. and may possibly be explained by different environmental conditions. However differences in the virulence of strains may also be important. Genetic analysis has revealed differences in the copy numbers of insertion sequence elements in African and European *M. mycoides* subsp. *mycoides* SC isolates (see Section 1.5.7.3.). African and European isolates were also differentiated by their ability to oxidise glycerol. Houshaymi *et al.* (1997) compared rates of glucose and glycerol oxidation in 14 *M. mycoides* subsp. *mycoides* SC strains. Washed cell suspensions were saturated with air, and oxygen uptake was monitored by measuring the decline in dissolved oxygen tension using an oxygen electrode. The ten Italian and Portuguese strains tested were unable to oxidise glycerol; however, the African and Australian strains oxidised glycerol with oxygen uptake rates of 88 to 540 % of those for glucose. Glycerol oxidation leads to production of hydrogen peroxide, a potential virulence factor (Houshaymi *et al.*, 1997), and this might account for mortality rate differences.

A mouse model to determine the virulence of *M. mycoides* subsp. *mycoides* SC strains, based on mycoplasmaemia, was developed by Dyson and Smith (1975). This model has severe limitations as infection is not apparently established and only mycoplasma survival is tested. Nevertheless, March and Brodie (2000) used the mouse model to suggest that a Botswanan *M. mycoides* subsp. *mycoides* SC isolate which had unusual growth characteristics and lacked an IS*1296* element was of reduced virulence! However, close examination of their data shows marked inconsistencies in controls, compared to those of Dyson and Smith (1975).

1. 5. 4. PREVALENCE AND ECONOMIC EFFECT

Early classical writings suggest CBPP existed in the ancient world (Provost *et al.*, 1987). During the 16th Century, CBPP appeared confined to the Alps and Pyrenees of Europe. It was first reported in Britain by Barker in 1736, although it was not until 1773 that Albert de Haller recommended mass slaughter for its

control (Egwu *et al.*, 1996). In 1765 Bourgelat fully described the clinical signs of CBPP, (Provost *et al.*, 1987) but CBPP was not properly distinguishable from other diseases until the end of the 19th century, when the organism now known as *M. mycoides* subsp. *mycoides* SC was isolated as the causative agent (Nocard and Roux, 1898).

The disease spread rapidly through mainland Europe in the early 19th Century due to increased cattle trading. Britain became reinfected in 1840 following the introduction of a bull from the Netherlands. The disease then spread to Scandinavia, USA, Australia and South Africa in the mid 1850's, followed by New Zealand, India, China, Mongolia, Korea, Hong Kong and Japan in the late 19th and early 20th Centuries (Egwu *et al.*, 1996). Hutyra *et al.* (1938), estimated the cost of the disease in Britain in the 1860's at £2 million per annum, and in Australia for the same period at £8.5 million per annum (Egwu *et al.*, 1996). Some data of cattle losses incurred are given in Table 1. 9.

TABLE 1. 9. CATTLE LOSSES DUE TO CBPP IN EUROPE IN THE 19TH CENTURY

	COUNTRY		
	NETHERLANDS	**UNITED KINGDOM**	**FRANCE**
Years	1833 - 1865	1841 – 1866	1827 - 1846
NUMBER OF CATTLE LOST	100,000	1,187,000	212,000

Data from Blancou (1996).

According to Hutyra *et al.* (1938), CBPP was eradicated from Western Europe by the beginning of the 20th Century, although sporadic outbreaks continued on the French/Spanish borders until the 1920's. However a thorough examination of the literature shows that outbreaks have been reported in each decade of the 20th Century (Nicholas *et al.*, 2000a). In the 1930's outbreaks were reported in Spain, Poland and Russia, while in the 1940's the disease was still continuing in Iberia

(Curasson, 1942). Sporadic outbreaks of CBPP occurred in Portugal in 1953, 1954, 1958 and from 1983 to 1999 (Table 1. 10). The disease was considered endemic in North-Western parts of Portugal; however only twelve cases were reported in 1998 and just one in 1999. CBPP was also reported by: Spain, 1961 and 1989 to 1998; France, 1980, 1982 and 1984 (Provost *et al.*, 1987); and Italy, 1990-1993.

In Italy the disease spread quickly and 94 outbreaks occurred in three years (Bashiruddin, 1996). Abattoir surveillance and the control of animal movements, linked to serological monitoring and slaughter of infected and contact animals, effectively controlled the disease and officially no cases have been reported since 1993; however Stradaioli *et al.* (1999) reported detecting *M. mycoides* subsp. *mycoides* SC in bull semen from Italy in 1994. Italy was officially declared free of CBPP in 1996.

TABLE 1. 10. NOTIFIED OUTBREAKS OF CBPP IN PORTUGAL BETWEEN 1985 AND 2000

Year	Total number of CBPP outbreaks
1985	884
1986	484
1987	723
1988	794
1989	1317
1990	1123
1991	1233
1992	829
1993	165
1994	69
1995	23
1996	26
1997	64
1998	12
1999	1
2000	0

Data modified from Regalla *et al.* (1996a) and Anon (1997-2000)

In Eastern Europe, CBPP existed in Russia, Romania, Poland, Austria and Germany until the late 1930's; however the current situation is unclear. Hungary is thought to be free of CBPP following a survey during 1998-1999 that tested more than one thousand sera, and nearly one thousand lung samples and nasal swabs using serological, cultural and PCR techniques (Stipkovits *et al.*, 2000a).

Little data exists for the disease in Asia and the Middle East, as surveillance systems are ineffective or non-existent. However, sporadic outbreaks are believed to occur in the Yemen, United Arab Emirates, Saudi Arabia, Qatar, Kuwait, and Lebanon mainly as a result of cattle importation from Africa. Pakistan,

Nepal and Jordan are similarly thought to be affected (Egwu *et al.*, 1996). The disease also appears to be present in an area from the west of China towards Thailand and Vietnam and covering Mongolia, Tibet, Bangladesh, Sichun, Bhutan, Myanamar, Burma, Kampuchea and Assam (Lefèvre, 1991; Ter Laak, 1992). A report by AnZu *et al.* 1996 gives details of three large outbreaks of CBPP in China in 1956, 1959 and 1964. Since then, serological surveillance in 16 counties in Henan, China during 1988-92 showed 1.19% of cattle (4,555 out of 380,000) were seropositive. A successful eradication scheme was conducted and the province was declared CBPP free in 1994. The disease entered Australia on two occasions in 1858 and 1922. The disease was eradicated by a combination of slaughter and vaccination. No cases of CBPP were reported after 1967 but vaccination continued until 1972 (Anon, 1972) with freedom from CBPP being officially declared in 1973 (Blancou, 1996).

CBPP nearly disappeared from the African continent following a campaign for the control of CBPP in the 1960's and 1970's (Masiga *et al.*, 1996). However, the disease is currently widespread, and is thought to affect at least 27 countries (Masiga *et al.*, 1998) (Table 1. 11). Many factors have affected the failure to control the disease. Nomadism and the trekking of trade cattle together with economic weakness and political and military disturbances have made movement restrictions and other control measures difficult. There is also a lack of diagnostic tools and an epidemiological surveillance network. Recent vaccination campaigns have been generally unsuccessful (Rweyemamu and Benkirane, 1996).

Kenya, Uganda, Mali, Rwanda and Tanzania which were free of CBPP are now experiencing endemic disease and Angola, Benin, Burkina Faso, Cameroon, Ethiopia, Ivory Coast, Ghana, Guinea, Namibia, Nigeria, Somalia, Togo, Zaire and Zambia are infected (OIE, 1993; Egwu *et al.*, 1996; Masiga *et al.*, 1996; 1998). In addition it is believed that Central African Republic, Congo, Democratic Republic of Congo, Djibouti, Eritrea, Somalia and Sudan are also infected (Masiga *et al.*, 1998). Since 1995, Mauritania has been experiencing severe CBPP epidemics which puts the neighbouring country of Senegal at risk (Rweyemamu and Benkirane, 1996).

The economic impact is high in those countries where the disease is endemic and CBPP is now the most economically important cattle disease in Africa (OIE, 1995). The direct losses result from mortality, reduced milk yield, vaccination campaign costs, disease surveillance and research programmes. The indirect costs are mainly due to the chronic nature of the disease and include loss of weight and working ability, delayed marketing, reduced fertility, losses due to quarantine and consequent reduced cattle trade (Egwu *et al.*, 1996). The Botswanan veterinary authorities in 1995 decided to depopulate the whole district of Ngamiland, slaughtering 220,000 cattle. Compensation alone was estimated at $30 million. This was successful in eradicating the disease in Botswana, being officially declared free of CBPP in 2000. In Nigeria the losses of cattle in 1991 were estimated to be in excess of $1.5 million. Tanzania had outbreaks between 1990-1995 that resulted in the deaths of 14,000 cattle valued at over $1 million.

TABLE 1. 11. REPORTED OUTBREAKS OF CBPP IN AFRICA 1987 – 1997

Country	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997
Benin	+*	+	+	4		+	5	12	3	8	7
Burkina	10	21	7	9	4	6	8	7	29	28	29
Cote D'Ivoire	7	9	14	18	16	9	12	7	12	11	10
Ghana	10	6	4	5	2	+	2	3	1	5	50
Guinea	?	105	49	38	82	49	20	17	48	30	+
Mali	4	5	15	37	12	15	20	21	11	12	6
Mauritania	0	0						0	6	5	+
Niger			0	5	8	13	4	8	3	8	9
Nigeria	49	46	114	48	55	27	17	18	6		+
Sierre Leone	0	0	0	0	+	+	0	0	0	0	0
Senegal	0	0	0	0	0	1	0	0	0	0	0
Cameroon					1	1	1		0	1	2
Chad	1	+	0	0	1	0					+
Burundi										+	+
Eritrea						+	+	+	0	0	0
Ethiopia	96	1	+	3	6	1	+	10	14	55	18
Kenya	6	2	15	39	24	11	10	6	6	3	+
Rwanda								+	+	+	+
Somalia	3	2									+
Sudan	+	+	+	4	0	0	0	0	0	0	0
Tanzania			0	+	1	+	1	3	259	41	57
Uganda	49	56	4	11	28	5	3	+	18	6	14
Angola	1	22	26	+	15	21	19	38	149	50	+
Botswana	0	0	0	0	0	0	0	0	45	0	0
Namibia	4	6	0	1	2	3	2	7	25	47	26
Zambia	0	0	0	0	0	0	0	0	0	0	2

Blank spaces in the Table indicate that no reports were submitted.

+*, CBPP reported but numbers of outbreaks not known.

Table based on Masiga *et al.* (1998) and derived from OIE World Animal Health Reports, FAO-OIE-WHO Animal Health Yearbooks and PARC reports.

In Europe the cost of controlling the Italian outbreak of 1990-1993 was over 28 million ECUs. The cost of the eradication campaign lasting more than 15 years in Portugal is probably incalculable. Costs are also incurred by countries such as Switzerland that are considered free of the disease but recently reactivated a surveillance system to provide documentation of their disease free status (Stärk *et al.*, 1995).

1. 5. 5. METHODS OF CONTROL

Identification of affected animals using serological testing and observation of classical lesions in cattle at the abattoir is essential so that control methods can be implemented. The most effective way to control the disease is by elimination of affected and contact animals along with restriction of cattle movement in affected areas. However, this option is not practical in most developing countries where containment and vaccination are preferred. Vaccination may provide protection for up to 1 year (Egwu *et al.*, 1996), but to be effective all animals within designated areas need to be vaccinated. Antimicrobials have not been effective in treating or controlling the disease. In Africa, their use has suppressed symptoms, probably aiding the spread of the disease (Egwu, personal communication). However, in view of the worsening situation, a FAO Emergency Prevention System (EMPRES) workshop recommended that research into the role of chemotherapy should be pursued (Rweyemamu and Benkirane, 1996). Recent *in vitro* minimum inhibition concentration (MIC) studies (Mazzolini *et al.*, 1997) showed *M. mycoides* subsp. *mycoides* SC was sensitive to chloramphenicol, enrofloxacin, erythromycin, lincomycin, oxytetracycline, spectinomycin, spiramycin, tiamulin, tilmicosin and tylosin (see Chapter 3).

Guidelines for control of CBPP have been issued by the OIE (1993) with the aim of eradicating CBPP, initially from endemic regions, and eventually worldwide. There is a three-stage process for a country to be declared free of CBPP. Stage one is 'provisional freedom from disease', which is made by the national chief veterinary officer based on clinical evidence available. This would lead to increased surveillance for the disease including meat inspection by the veterinary service. In addition the use of vaccination would be discontinued. After two years the second stage of 'freedom from disease' would be confirmed subject to criteria including

continued absence of clinical disease, the adoption of adequate surveillance and disease reporting systems and effective measures to prevent re-introduction of the disease. After two more years, an OIE expert panel may declare 'freedom from infection' subject to second stage criteria being met and an undertaking for continued monitoring and reporting of disease. The implementation of these guidelines is feasible in Europe, but probably not yet possible in most of the affected African countries (FAO, 1999).

1. 5. 5. 1. VACCINES

The only proven vaccine effective against CBPP is live broth culture of attenuated strains. The minimum vaccinating dose for the $T_1$44 vaccine was estimated to be 10^7 cfu by Gilbert and Windsor (1971). This is the standard adopted for the lyophilised PANVAC vaccine. To allow for loss in viability during lyophilisation, storage, transportation and usage of the vaccine, high initial viable counts are required (Litamoi, 1998). Vaccine quality control is therefore an essential prerequisite for any CBPP control programme. Leitch and March (2000), suggested that the inverse of the absorbance (A_{540}) of broth cultures using a 0.006% phenol red indicator could be used to estimate the titre of $T_1$44 vaccine, thus improving vaccine quality control methods. This method was previously described by Meur *et al.* (1989) for use with *M. mycoides* subsp. *capri.* However, batches of $T_1$44 vaccine may also vary in effectiveness as this vaccine strain has been found to consist of an antigenically mixed population (Rweyemamu *et al.*, 1994). A variation in the genome sequence of a haemolysin-like protein has also been noted amongst different sources of the vaccine (Chirnside *et al.*, 1998). It has been suggested that this may account for the apparent failure of the Botswana vaccine (Masupu, 1999), but further work is required to establish this. Currently the OIE (OIE, 1996) only recommend strain $T_1$44, and its streptomycin resistant variant, for preparing CBPP vaccines. Strain $T_1$44 still retains some measure of residual virulence with subcutaneous inoculation causing unpredictable post vaccinal reactions which may lead to death in a small number of animals (Litamoi, 1998). Table 1. 12. summarises the key characteristics of the vaccine strains in recent or current use. One of the major problems with all vaccine strains is the short-lived immunity, up to 1 year (Tulasne *et al.*, 1996).

There is clearly a need for a new vaccine, which is stable, risk-free, cheap and gives long-term protection. Abusugra and Morein (1996) described a CBPP experimental immunostimulating complex (ISCOM) vaccine that was used to stimulate a T helper cell type 1 (Th 1) response. Antibody responses were detected in four test cattle after primary experimental inoculation, and titres rose 10 fold after a booster inoculation. In BALB/c mice, subcutaneous and intranasal administration of the ISCOM produced high mucosal and systemic antibody responses (Abusugra and Morein, 1999). However, the vaccine has yet to be successfully tested under field conditions.

TABLE 1. 12. CURRENT OR RECENTLY USED CBPP VACCINES

VACCINE STRAIN	ADVANTAGES	LIMITATIONS	AVAILABILITY
KH3-J & KH3-J SR*	Inocuous	Duration of immunity (six months)	Production stopped because of poor immunogenicity.
$T_1$44	Potency (immunity for 1 year)	May cause severe side-reactions	Senegal, Mali, Sudan, Kenya, Nigeria
T_1SR*	Innocuous, may be combined with rinderpest vaccine	Effective dose may be higher than $T_1$44.	Senegal, Mali, Niger, Cameroon, Chad, Ethiopia, Botswana, Namibia, South Africa (fermenter grown)

Table adapted from Rweyemanu and Benkirane (1996).

* SR strains are streptomycin resistant. This enables differentiation of clinical isolates during vaccination campaigns.

1. 5. 6. IMMUNOLOGY

Cattle that have recovered from CBPP are resistant to further infection (Hudson, 1971), which suggests that they must mount a specific protective immune response. The majority of naturally infected cattle with lesions have a detectable antibody response, but there is no relationship between the severity of lesions and

the complement fixing antibody titre (Bygrave *et al.*, 1968). Cattle infected by contact with experimentally infected animals showed similar CFT and ELISA IgM profiles over a 4-month period; seroconversion took place at 8-10 weeks and persisted for about ten weeks (Le Goff and Lefèvre, 1989). An IgG response was detected by ELISA one week later than the IgM response, and remained detectable for more than 10 weeks. In experimental infections, Miserez *et al.* (1996) showed that mycoplasmas were generally excreted before a serological reaction was detected. An experimental infection by Pini *et al.* (1999), using a European *M. mycoides* subsp. *mycoides* SC isolate, produced detectable immune responses (see Chapter 6, this thesis) although there were few clinical signs of disease.

Cell-mediated immune responses also seem necessary for the prevention and resolution of CBPP (Nicholas and Bashiruddin, 1995); however, few studies have been reported. Windsor *et al.* (1972) demonstrated oedematous immediate and delayed-type hypersensitivity reactions in cattle after inoculation with antigens prepared from *M. mycoides* subsp. *mycoides* SC cultures. Dedieu *et al.* (1998) established that experimentally infected zebus developed a specific T-cell immune response; this used specific memory T-lymphocytes since immunological memory of specific *M. mycoides* subsp. *mycoides* SC antigens was retained. Investigations using a gamma-interferon assay failed to detect a T-cell response in sheep challenged with *M. mycoides* subsp. *mycoides* SC. However, it did detect a response in experimentally infected cattle, giving results that correlated with other serological indicators. Further work would be needed to develop the assay as a test for infection (Durán *et al.*, 2000).

Studies with other mycoplasmas suggest that local immunity is very important in host defence. In man there is a relationship between IgA antibody in respiratory secretions and immunity to *M. pneumoniae* (Krause and Taylor-Robinson, 1992); and in cattle resistance to *M. bovis* was related to specific IgG in lung washings (Gourlay and Howard, 1982). Thus, the location of *M. mycoides* subsp. *mycoides* SC, primarily on the mucous membrane of the respiratory tract may explain the poor correlation between serum antibody concentration and resistance to infection.

1. 5. 7. DIAGNOSTIC METHODS

1. 5. 7. 1. SEROLOGICAL TESTS

Many serological tests have been described for the detection of *M. mycoides* subsp. *mycoides* SC-specific antibodies or antigen. These include slide agglutination (Priestly, 1951; Turner and Etheridge, 1963), complement fixation (CFT) (Campbell and Turner, 1953), agar gel precipitation (Gourlay, 1965), passive haemagglutination (PHA) (Chima and Onoviran, 1982), growth inhibition (Clyde, 1983) immunofluorescence (Del Giudice *et al.,* 1967), metabolic inhibition (Purcell *et al.,* 1966; Taylor-Robinson *et al.,* 1966), enzyme linked immunosorbent assay (ELISA) (Onoviran and Taylor-Robinson, 1979), Western blotting (Nicholas *et al.,* 1996), and immunocytochemical tests (Ferronha *et al.,* 1988; Scanziani *et al.,* 1991; Ayling *et al.,* 1998; Bashiruddin *et al.,* 1999).

Detection of the immune response to CBPP infections in cattle is the major means of diagnosis and hence control of the disease. The preferred 'classical' method is the CFT, although it is relatively laborious and insensitive. This test relies on antigen-antibody complexes fixing complement. A positive reaction is visualised by the failure of antibody-sensitised-sheep-erythrocytes to lyse in the presence of antigen-treated test serum because complement has been fixed. There is little room for error in this test, as any reaction at 1/10 dilution of sera or above is considered positive.

Amongst the newer serological methods, ELISA techniques have received the most attention. The typical format is an indirect ELISA, with the antigen immobilised on a plastic surface. The antigen is reacted with a dilution of test bovine serum, which is then reacted with an anti-bovine immunoglobulin conjugated to an enzyme. Bound enzyme activity is then detected using a chromogenic substrate. The ELISA is usually sensitive and easy to use. The first ELISA for CBPP (Onoviran and Taylor-Robinson, 1979) used a whole cell antigen, which successfully distinguished between uninfected and naturally infected or vaccinated cattle, although background levels were high. Le Goff (1986) showed clearer differentiation using a sonicated antigen. Comparisons of the ELISA, the CFT and the PHA test for detecting antibody responses in experimentally infected

cattle showed the ELISA to be more specific (Le Goff, 1986). However, in addition to detecting antibody to *M. mycoides* subsp. *mycoides* SC, positive results were also obtained for *M. mycoides* subsp. *mycoides* LC and bovine serogroup 7 antisera (Poumarat *et al.,* 1989). Le Goff and Lefèvre (1989, 1990) showed complement-fixing antibodies were detectable a few days before those detected by ELISA, but in contrast to CFT titres, positive ELISA titres were maintained throughout the *M. mycoides* subsp. *mycoides* SC excreting stage and for a period after. Other workers have used whole cell antigens treated with Tween 20, which removes some of the cross-reacting antigens making the test more specific (Ayling *et al.,* 2000).

The use of monoclonal antibodies to produce a specific ELISA or other tests for detecting antibodies to *M. mycoides* subsp. *mycoides* SC may be the best approach for the future. However, many monoclonal antibodies produced to date have not been specific to *M. mycoides* subsp. *mycoides* SC and have reacted with other members of the *M. mycoides* cluster (Mia *et al.,* 1993). A competitive(c) ELISA using a monoclonal antibody to an 80 kDa protein has been developed and is currently being evaluated (Le Goff and Thiaucourt, 1998; OIE, 1996). The test uses a high cut-off point to maximise specificity, but this reduces sensitivity. The cELISA detected antibody in experimentally infected cattle 4 to 8 days after the CFT although it detected infection for longer. It is expected that the OIE will accept the cELISA as an alternative to the CFT. However, its sensitivity is similar to that of the CFT (Yaya *et al.,* 2000) and therefore false negative reactions will continue to occur, enabling the persistence of disease.

Nicholas *et al.* (1996) used immunoblotting to analyse serum samples from the Italian CBPP outbreak. The analysis of sera using immunoblotting has a1so been described by Gonçalves *et al.* (1998), and it is this method which is used for confirmatory diagnosis of CBPP in Portugal. Briefly, a whole cell *M. mycoides* subsp. *mycoides* SC antigen is separated by electrophoresis on a 5 – 15% gradient gel and transferred to a nitrocellulose membrane (western blotting). The membrane is then used as a solid support for an antigen-antibody reaction similar to an ELISA. Sera are diluted 1 in 5 for the test, so many bands may be detected. It is the presence of specific antigenic bands at 110, 95, 62, 60, and 48 kDa that are considered definitive in confirming CBPP infection. Immunoblotting is used as the standard

confirmatory test for detecting *M. mycoides* subsp. *mycoides* SC antibodies in Portugal and it has been suggested that this should be extended internationally.

The development of tests for detection of specific antibodies may be aided by the cloning and expression of mycoplasma antigen genes in other organisms. This would enable their production in a standardised manner and in large quantity. Difficulties exist in cloning mycoplasma genes due to their unusual UGA_{trp} codon usage. However, Cheng *et al.* (1996) have cloned and expressed a specific immunogenic 72 kDa protein into *Escherichia coli.* Phage display systems have also been successfully used for 'shotgun cloning' of restriction fragments of *M. mycoides* subsp. *mycoides* SC resulting in mycoplasma polypeptides being displayed (Persson *et al.*, 1998). This 'shotgun cloning' may overcome some of the difficulties associated with cloning directly into *E. coli* and may help with the construction of genomic libraries for *M. mycoides* subsp. *mycoides* SC.

In the identification of *M. mycoides* subsp. *mycoides* SC cells, the growth inhibition test (Clyde, 1983) is most widely used. In this test, antiserum on filter paper discs is applied to the centre of inoculated plates and growth inhibition is detected after incubation. However, serological cross-reactions are observed with other members of the *M. mycoides* cluster. To overcome this Rodriguez *et al.* (1996a) used a monoclonal antibody-based sandwich ELISA that differentiated strains within the cluster; detection was most effective following enrichment and capture of cells in broth culture by antibody coated microtitre wells. However, this test could not distinguish between *M. mycoides* subsp. *mycoides* SC and *M. mycoides* subsp. *mycoides* LC.

1. 5. 7. 2. ISOLATION AND CULTURE

Mycoplasmas are dependent on their hosts for a large variety of organic nutrients such as vitamins, amino acids, fatty acids and lipids. In addition, *M. mycoides* subsp. *mycoides* SC will not grow in media lacking the preformed bases uracil, thymine, and guanine that it needs for nucleic acid synthesis (Razin, 1978).

The inorganic requirements of mycoplasmas are poorly known, but it appears that potassium, magnesium and phosphate ions are adequately provided by glassware contaminants and other growth-medium components (Rodwell, 1983). However, high concentrations of metallic ions, for example zinc, manganese, cobalt

and iron are inhibitory for *M. mycoides* subsp. *mycoides* SC (Rodwell and Mitchell, 1979).

Mycoplasma mycoides subsp. *mycoides* SC is a facultative anaerobe growing equally well in aerobic and anaerobic environments at a pH of 6.8-7.8. Many of the standard media available for mycoplasma culture are suitable for its growth (Bradbury, 1998b; Furr, 1998; Nicholas and Baker, 1998; Taylor, 1998). These typically contain thallium acetate and penicillin (or other antimicrobials) for isolation from clinical specimens. Recently Rice *et al.*, (2000b) optimised the concentrations of individual medium components including energy sources, peptone, yeast extract, serum, inorganic salts and buffers to develop a medium (PRM) giving high yields and growth rates of *M. mycoides* subsp. *mycoides* SC. *M. mycoides* subsp. *mycoides* SC is referred to as fermentative because it produces acid from glucose metabolism and this is of diagnostic value in the identification of clinical isolates. Fructose, *N*-acetylglucosamine and pyruvate may also be used as energy sources, but glucose supports higher growth rates (Miles *et al.*, 1986). Miles *et al.* (1996) found that *M. mycoides* subsp. *mycoides* SC strains were metabolically similar and readily distinguished from other members of the cluster by their inability to oxidise maltose, trehalose and (at low concentrations) mannose. Utilising a chromogenic α-glucosidase (maltase) substrate, Rice *et al.* (2000a) have developed a rapid test which differentiates *M. mycoides* subsp. *mycoides* SC and *M. capricolum* subsp. *capripneumoniae* from the other *M. mycoides* cluster members. Houshaymi *et al.* (1997) also demonstrated differences in substrate metabolism amongst *M. mycoides* subsp. *mycoides* SC isolates; in particular, African and Australian, but not European isolates, were able to oxidise glycerol.

1. 5. 7. 3. MOLECULAR TECHNIQUES

One of the most useful advances in recent times has been the development and application of polymerase chain reaction (PCR) technology for the detection and identification of *M. mycoides* subsp. *mycoides* SC. In the first PCR described, primers derived from the CAP-21 genome fragment were used. These amplified a 0.5kb region of both *M. mycoides* subsp. *mycoides* SC and LC strains, as well as strains of *M. mycoides* subsp. *capri.* However, amplicons from *M. mycoides* subsp. *mycoides* SC could be specifically identified by restriction enzyme analysis

with *Asn*I (Taylor *et al.*, 1992; Bashiruddin *et al.*, 1994a). Bashiruddin *et al.* (1994b) showed that the test could detect specific DNA from clinical materials such as lung, nasal mucous, pleural fluid and pulmonary tissue. A related PCR system can also detect all members of the *M. mycoides* cluster (Bashiruddin *et al.*, 1994a). These PCRs are useful for identifying cultured isolates and have been developed to include a colourimetric detection system (marketed as pleuroTRAP by AMRAD) useful for high throughput testing (Bashiruddin, personal communication). Although the tests are sensitive and specific, negative results have been obtained from seropositive animals suggesting that further work on sample handling may be required (Egwu *et al.*, 1996).

Further PCR systems have since been developed. Some also use the CAP-21 gene (Hoetzel *et al.*, 1994), whilst others are based on the ribosomal RNA gene in the belief that it will yield a more sensitive PCR, as there is a higher copy number of target molecules (Johansson, 1993; Miserez *et al.*, 1996). The use of nested or semi-nested primers has also been used with the aim of increasing sensitivity and specificity. Persson *et al.* (1999) described two PCR systems based on the two 16S rRNA genes; the first used laser-induced fluorescence to detect a unique sequence length difference between the genes in *M. mycoides* subsp. *mycoides* SC whilst the second relied on restriction endonuclease analysis.

Rawadi *et al.* (1995) used one 18bp primer (Mlip1) and a 17bp primer (Mlip4) for an arbitrarily-primed (AP) PCR that differentiated some of the *M. mycoides* cluster. The assay showed that the *M. mycoides* cluster could be divided into two groups: those with high polymorphisms and those with no variation. Specific polymorphic bands for members of species and subspecies were identified but only two distinct bands at 900bp and 100bp were obtained with the four *M. mycoides* subsp. *mycoides* SC strains tested, although a weak band was observed at 400bp with one strain. This test may therefore be useful for identifying *M. mycoides* cluster members, but the test would need careful optimisation. Although few *M. mycoides* subsp. *mycoides* SC strains were tested, AP-PCR shows little potential as an epidemiological tool for *M. mycoides* subsp. *mycoides* SC.

In situ PCR was used to confirm the presence of *M. mycoides* subsp. *mycoides* SC in paraffin embedded tissue from affected cattle (Camma *et al.*, 2000).

This technique combines the sensitivity of PCR with the possibility of locating a specific DNA sequence within intact cells or tissue sections. The technique is relatively slow and has little benefit for on-farm diagnosis, but is very sensitive enabling the confirmation of suspected CBPP when other tests have failed to detect the disease or given conflicting results.

The use of epidemiological tools to trace sources of infection and spread of disease are increasingly perceived as important by the authorities. In the UK, research on tuberculosis, salmonellosis and campylobacteriosis has led to the development of molecular tools to differentiate isolates for epidemiological tracing and possible determination of virulence factors. Molecular tools have included restriction enzyme analysis (REA), pulsed-field gel electrophoresis, low-molecular weight RNA profiles, amplified fragment length polymorphism and insertion sequence analysis. Insertion sequences encode only functions involved in their translocation, and transpose both within and between genomes. They are capable of promoting various types of genome rearrangement including deletions, inversions and replicon fusions (Mahillon *et al.*, 1999). Two *M. mycoides* subsp. *mycoides* SC specific insertion sequences, IS*1634* and IS*1296*, have recently been identified. About 30 copies of IS*1634* are present in the genome. It has a size of 1,872bp, including two 13-bp terminal inverted repeats and contains an open reading frame encoding a product of 533 amino acids (Vilei *et al.*, 1999). The IS*1296* is 1485bp long and has 30bp terminal inverted repeats and 18-20 copies are present on the chromosome. It has two open reading frames that could code for transposase-like proteins. The copy number of this insertion sequence has been used to investigate the epidemiology of isolates (Frey *et al.*, 1996) and led to the differentiation of African and Australian isolates from European isolates. This suggests that CBPP in Africa and Australia had different origins to that in Europe, or that the original strains are no longer found in Europe. Poumarat and Solsona, (1995) used restriction enzyme analysis (REA) on 27 *M. mycoides* subsp. *mycoides* SC isolates to obtain five different profiles with *Pst*I and three with *BamH*I. Twelve out of 15 European isolates had different profiles to those obtained with ten African isolates. The three isolates with the same profiles as the African strains all came from the Iberian Peninsula, but it was later shown that these isolates had been imported from

Africa for experimental work. The vaccinal strains T_1SR and KH_3J had a unique REA profile.

Pulsed-field gel electrophoresis (PFGE) has been used to construct genomic maps of some strains within the *M. mycoides* cluster (Pyle *et al.*, 1990) but it has not yet been used for epidemiological typing of *M. mycoides* subsp. *mycoides* SC isolates. Currently no reports of amplified-fragment length polymorphism fingerprinting of *M. mycoides* subsp. *mycoides* SC have been published; however this method has been successfully used for differentiating strains of other *Mycoplasma* species (Kokotovic *et al.*, 1999). Low-molecular weight RNA profile analysis of members of the *M. mycoides* cluster, clearly differentiated between *M. mycoides* subsp. *mycoides* SC and LC strains but did not reveal differences within isolates belonging to each group (Botelho *et al.*, 2000).

In the future, the development of molecular tests for *M. mycoides* subsp. *mycoides* SC may be aided by the publication of the complete genome sequence for strain PG1. This sequence is almost complete and is expected to be available by the year 2002 (Johansson, personal communication).

1. 5. 7. 4. DIAGNOSTIC METHODS IN THE UK

The United Kingdom is currently free of CBPP and it is essential to maintain CBPP-free status for animal health and economic reasons including trade with other CBPP free countries. However, the country is at risk from imported cattle carrying the disease. Imported cattle from suspected countries are screened serologically using the CFT (OIE, 1996); however asymptomatic animals in the early stages of infection and chronically infected animals may not have detectable antibodies (Provost *et al.*, 1987). The CFT detects only 70 % of chronically infected animals and may give false positive results (Stärk *et al.*, 1994). Thus, positive or suspect positive sera are further tested using western blotting for the presence of *M. mycoides* subsp. *mycoides* SC-specific protein antigens (Nicholas *et al.*, 1996).

Occasionally, field workers, veterinarians or abattoir workers may suspect an animal has CBPP. Abattoir surveillance by experienced meat inspectors is a key component of CBPP-detection. It is recommended by the OIE (OIE, 1996) and played a major role in the control of Italian outbreaks of the disease (Regalla *et al.*, 1996a). In the UK suspect cases are reported to the Department of Environment,

Food and Rural Affairs (DEFRA) and testing is undertaken immediately at the Veterinary Laboratories Agency (Weybridge). Many different samples are submitted, including blood, nasal swabs, pleural fluid and some lung tissue; however not all of these may be available as the animal may still be alive or samples may be lacking.

Culture and identification of *M. mycoides* subsp. *mycoides* SC is necessary to confirm the disease but this may take up to two weeks. Difficulties may also arise with the overgrowth of the sample by other bacterial species and mollicutes such as *Acholeplasma laidlawii* that are frequently found in CBPP affected cattle. At VLA, Eaton's medium (Nicholas and Baker, 1998) is used with additional antimicrobials (see Chapter 2, Section 2.) for primary isolation and several dilutions of the sample are made to avoid overgrowth. Recently, Abu-Amero *et al.* (1996) incorporated nisin into mycoplasma media to inhibit the growth of *Acholeplasma* species and this may be adopted at VLA. Identification of isolates of *M. mycoides* subsp. *mycoides* SC would be based on their sensitivity to digitonin; ability to ferment glucose; and sensitivity to growth inhibition by hyperimmune rabbit antiserum (Provost *et al.*, 1987). In addition, the PCR of Bashiruddin *et al.* (1994b) is available for the detection of specific *M. mycoides* subsp. *mycoides* SC DNA. The use of PCR provides a more rapid and specific method of detecting the presence of the organism and confirming the identification of suspect isolates.

1. 6. AIMS OF THE THESIS

This thesis is concerned with the diagnosis and control of two major bovine mycoplasma diseases, CBPP and *M. bovis* infections of cattle. The importance of these diseases has been described in sections 1. 4. and 1. 5. *M. bovis* is currently the most important disease-producing mycoplasma of cattle in the UK and CBPP is a major threat to the UK and European cattle industry. The current areas of difficulty in the detection of *M. bovis* infections are:

(i). Intermittant shedding of the organism, which reduces the sensitivity of detection by culture;

(ii). The inability to detect a specific immune response in calves less than three months of age;

(iii). The presence of variable surface proteins (vsp's);

(iv). The potential for serological cross-reactions of *M. bovis* and *M. agalactiae.* This is not thought to be a serious problem, as *M. bovis* is associated almost exclusively with cattle and *M. agalactiae* with sheep and goats. However, there have recently been a number of reports of mycoplasma isolates from atypical hosts including *M. bovis* from goats (Chima *et al.*, 1986) and also from man (Madoff *et al.*, 1979). Thus, a test guaranteeing differentiation of *M. bovis* and *M. agalactiae* would be of value.

In relation to *M. bovis* disease control, vaccines are in the early stage of development. Since, *M. bovis* is endemic and there is no attempt to control its spread by movement restriction orders, the only effective control measure in the UK is antimicrobial treatment. However, at the start of work reported in this thesis there was no published information giving antimicrobial resistance patterns of UK *M. bovis* isolates.

The aim of the *M. bovis* work conducted in this thesis was to:

(i). Develop a specific PCR, enabling the sensitive detection of organisms in culture and clinical specimens.

(ii). Determine the antimicrobial sensitivity pattern of UK *M. bovis* isolates. In order to determine the sensitivity of a large number of strains to several antimicrobials, a microtitre plate method was developed for use with mycoplasmas. The method was used to determine both minimal inhibitory (MIC) and minimal mycoplasmacidal (MMC) concentrations.

In the UK, the major concern regarding CBPP is the presence of the disease in imported animals. The problems of diagnosis in such animals are:

(i). Test sensitivity;

(ii). Test specificity;

(iii). The presence of asymptomatic carrier animals that harbour the disease without developing a detectable immune response;

(iv). The presence of animals at an early stage of infection.

Treatment of CBPP infected animals is not an option in Europe, where the disease is controlled by the slaughter of all infected and contact animals. In African countries where the disease is endemic, controls include the use of vaccines and

restriction of movement. However, these are currently ineffective (see Section 1.5). Thus, chemotherapy may offer an alternative option (Rweyemamu and Benkirane, 1996).

A major initial aim of the work on *M. mycoides* subsp. *mycoides* SC in this thesis was to improve the sensitivity and specificity of serological tests. The approaches adopted included comparing current serological test methods with culture, PCR and gross pathology of animals from a CBPP outbreak in Northern Portugal. Work was also conducted on an indirect ELISA using whole cell and tween 20 treated cells as antigens and a novel latex agglutination test. It was thought that this test would be of limited value in the UK, but it appeared to offer several advantages for use in African countries. Thus, optimisation and evaluation of the test was carried out. In addition, MIC and MMC values for various *M. mycoides* subsp. *mycoides* SC strains was determined as described for *M. bovis* above.

Finally, in work covered during the early stages of this thesis, the molecular (16S rRNA) characterisation of potentially novel mycoplasmas from a perigrine falcon and an ostrich was carried out. The methods used were of relevence to the development of molecular diagnostic techniques and the identification of novel mycoplasmas and are referred to in the introduction and discussion sections of the thesis. The work was not included in the main body of the thesis, which was dedicated to *M. bovis* and *M. mycoides* subsp. *mycoides* SC diagnosis and control, but is briefly summarised in Appendix 1.

CHAPTER 2

2. 1. DEVELOPMENT AND VALIDATION OF A PCR FOR THE DETECTION OF *MYCOPLASMA BOVIS*

2. 1. 1. INTRODUCTION

Mycoplasma bovis is endemic in Britain, causing pneumonia, arthritis, mastitis, subcutaneous abscesses, keratoconjunctivitis, meningitis and infertility (Pfützner and Sachse, 1996); more recently it has also been implicated as a cause of abortion (Byrne *et al.*, 1999). The significance of *M. bovis* as a pathogen of cattle was discussed in Section 1.4. Control of the disease is difficult, but correct diagnosis is important to differentiate it from other more readily treated diseases. Current diagnosis is dependent on either serological testing by ELISA (Nicholas *et al.*, 2000b) or by culture and identification of *M. bovis.* Currently, the VLA annually receives 4000 sera for ELISA and 400 cultures from possible cases of *M. bovis* infection. Thus efficient diagnostic methods are clearly needed.

2. 1. 2. ISOLATION AND IDENTIFICATION OF M. BOVIS

Diagnosis of disease caused by *M. bovis* on the farm is difficult, and *M. bovis* is usually suspected only if treatment with antimicrobials for other bacteria is unsuccessful. Serological testing by ELISA may indicate that an animal has had contact with *M. bovis*, but very young calves that are most likely to die from *M. bovis* pneumonia do not show a detectable immune response. Culture and identification of the causative organism is the main method of confirming the presence of disease. However, animals with subclinical or chronic disease only harbour a small number of organisms that are shed intermittantly (Mattsson *et al,* 1991). Thus, the success of cultures from nasal swabs may be limited. Culture from bronchiolavage samples is generally more successful, but this is not done routinely as it is a difficult method for routine farm use. Samples taken from pneumonic lungs at post-mortem are most likely to enable *M. bovis* isolation and the gross pathology is also indicative of the disease (see Section 1.4).

The culture of *M. bovis* requires a specialised mycoplasma growth medium. Eaton's broth medium (see section 2.2.2.) is used at VLA (Weybridge) and for

primary isolation selective inhibitors such as thallium acetate and penicillin are included. However, although *M. bovis* is a relatively rapid growing mycoplasma, it may be outgrown by other less important bacteria and *Acholeplasma laidlawii.* Thus it may be necessary to filter initial broth cultures through 0.45 μm filters to remove bacterial contaminants and then re-culture the filtrate. Initial growth usually takes 2-3 days, although if filtration steps are required the isolation will take much longer.

Isolates are first confirmed as being *Mycoplasma* by demonstrating growth inhibition in the presence of digitonin or polyanetholsulphic acid. Impregnated discs are placed on an agar plate seeded with the culture and plates are incubated at 37°C (in 5% CO_2 in air) and examined daily for growth. Cultural characteristics are then determined. *M. bovis* is suspected when a cattle isolate does not ferment glucose or hydrolyse arginine, and produces a film on the surface of broth or agar media. Confirmation of identification is carried out either by an indirect immunofluorescent antibody test (IFAT) on colonies, or by growth or film inhibition tests. These methods usually rely on anti-*M. bovis* polyclonal rabbit antiserum and are described in detail by Poveda and Nicholas (1998).

In growth inhibition tests, optimum results are obtained if several dilutions of culture are tested, as heavy inoculations may lead to overgrowth of inhibition zones. This is therefore expensive on medium and specific antiserum, especially if the culture is tested against more than one mycoplasma species. Another disadvantage of the growth inhibition test is the time taken to obtain a result; for *M. bovis*, isolates two days incubation are required before growth of the organism becomes visible and a result may be recorded. The film inhibition test for *M. bovis* is similar to the growth inhibition test, except that it is carried out using a medium containing egg yolk, which is rich in phospholipids, thereby encouraging film development (Poveda and Nicholas, 1998).

The IFAT requires growth of the organism on agar medium. Sections of medium with colony growth are then excised from the agar plate and placed on a microscope slide. Negative control and mycoplasma species-specific antisera are added to different agar blocks from the same mycoplasma isolate and incubated. Blocks are then washed in phosphate buffered saline (PBS) and fluorescein

isothiocyanate (FITC) conjugated antiserum is added. After incubation, there is a further washing step in PBS. Finally colonies are examined for fluorescence using an ultra violet light microscope. The method is very labour intensive and critically dependent on washing in PBS to reduce non-specific background fluorescence. Cross-reactions between species occur and correct interpretation of the test requires an experienced investigator. Where *M. bovis* growth leads to film development, background levels of fluorescence are high making the test difficult to read. A further potential problem with the IFAT, and growth and film inhibition tests, is surface antigen variation. Behrens *et al.* (1996) reported that the major immunogenic proteins of *M. bovis* include variable surface proteins (vsp's) that undergo dynamic and spontaneous changes in size and expression. Conventional tests may therefore occasionally fail to identify *M. bovis* isolates because of this antigenic variation (Rosengarten *et al.*, 1998). For example, in 1996, two bovine mycoplasma isolates submitted to VLA (Weybridge) could not be identified by serological techniques. Subsequently, they were identified as atypical *M. bovis* by Dr Konrad Sachse, BgVV, Jena, Germany. He showed using PCR with *vsp* gene specific primers and Southern blot hybridization, that they lacked typical *vsp* gene components. Immunoblotting using vsp specific monoclonal antibodies also demonstrated that the isolates did not possess the 'classical' *vsp* system as described for the type strain PG45.

2. 1. 3. MOLECULAR TYPING METHODS

The lengthy procedures required to isolate, biochemically characterise and serologically confirm isolates as *M. bovis*, make it extremely unlikely that a farmer will receive a diagnosis in less than two weeks; in practice, it may take longer. Thus, alternative methods are needed that will decrease identification time and reduce the use of expensive antiserum.

The development of molecular typing tools for microorganisms began in the 1980's in the form of probes produced from genes or nucleotide sequences specific to a group of species, a single species or a certain strain within a species. Probes improved the detection and identification of infectious agents in a wide range of clinical material. These probes hybridised to the DNA or RNA in a sample to confirm the presence of the target organism (Woodward, 1989). Probes were

originally labelled with radionucleotides but safer labelling systems, using for example digoxigenin, are now available (Boehringer Mannheim, Germany).

More recently probes have been replaced by polymerase chain reaction (PCR) tests which are more sensitive and less labour intensive. The PCR enables selective amplification of a specific region of a DNA molecule so long as the sequences at the borders of the region are known. In the technique two short chemically synthesised oligonucleotides are annealed to the DNA molecule, one to each strand of the double helix, which delimit the region to be amplified. A heat stable polymerase enzyme is added to the now primed template DNA so that it synthesises new complementary strands. On heating, the newly synthesised strands detach from the templates so that when cooled more oligonucleotides anneal to their respective positions and a second round of DNA synthesis can begin. This cycling process is repeated 30 - 40 times resulting in a single DNA sequence being amplified over 10^9 times within a few hours. PCR can detect minute amounts of specific DNA in a sample or culture (Newton and Graham, 1994). Other than a thermocycler, PCR requires limited specialist equipment, but a clean laboratory area (cabinet) dedicated to PCR is essential to avoid DNA contamination.

The PCR has enabled major advances in the diagnosis and identification of organisms including the *Mollicutes*; however in the development of tests, careful optimisation of reaction conditions and sample treatment is required. For example, in some samples DNA has to be extracted because of the presence of undefined inhibitors. One of the disadvantages of a specific PCR in the diagnostic laboratory is the need for many PCR systems to cope with the range of species to be identified. This has been partly overcome by using primers from the conserved regions of a specific gene that can amplify target DNA from a group of related species. The amplicon then undergoes cleavage by specific restriction enzymes that will give different size fragments depending on the species (Fan *et al.*, 1995).

Many PCRs are based on the 16S rRNA gene that has approximately 1500 nucleotides with highly conserved regions but also many variable regions that are species specific. The sequences of 16S rRNA genes may be determined following PCR reactions using 'universal primers' based on the highly conserved regions of the 16S rRNA gene. Many 16S rRNA sequences are known and are

available from databanks, thus enabling the design of species-specific primers. Whole 16S rRNA gene sequences may also be used to identify organisms. Amongst *Mollicutes* 16S rRNA sequences are known for representative species of all genera (Johansson *et al.*, 2000). In addition complete sequences for the type strains of all validly described *Mycoplasma* species (currently 102) are known or will be available by the end of the year 2001 (Johansson *et al.*, 2000).

The sequencing of the 16S rRNA gene opens a new route to the identification of mycoplasmas occurring in atypical hosts and the recognition of novel isolates as potential new species. Currently serological testing is a major feature among the requirements for naming a new *Mollicutes* species. Cells of the potential new species must be tested by GIT or IFAT against the specific antiserum of all known species, and the specific antiserum of the novel species must similarly be tested against cells of all known species. Since there are already 102 recognised *Mycoplasma* species, few, if any, laboratories have all the required resources. However, 16S rRNA sequences cannot be relied on as the sole means of identifying new species, partly because of the confusion that would arise from the occurrence of polymorphisms. It has been proposed (Johansson *et al.*, 2000) that 16S rRNA sequencing could be used to determine which phylogenetic cluster a novel isolate belongs to and that serological analyses need only be performed with the members of that cluster. This would limit the number of species for serological comparison to a maximum of 22 (Johansson *et al.*, 2000). This proposal has been put to the Subcommittee on Taxonomy of Mollicutes and is currently awaiting their approval. The strategies used to obtain rRNA sequences and their application to the identification of unusual isolates from sheep and potential new species from peregrine falcons and ostrich are described in Appendix 1.

2. 1. 4. PCR METHODS FOR M. BOVIS

Bovine pneumonia caused by mycoplasmas has a major economic impact on cattle production worldwide (Rebhun *et al.*, 1995). The major causes are *M. bovis* which is endemic in the UK (Section 1.4.) and *M. mycoides* subsp. *mycoides* SC (Section 1.5.) which is exotic to the UK but causes enormous losses particularly in African countries. PCRs for identifying *M. mycoides* subsp. *mycoides* SC (Bashiruddin *et al.*, 1994a) have been used in the VLA (Weybridge) laboratory

for several years; a similar test for *M. bovis* would speed up diagnosis enabling control measures to be implemented rapidly.

Chávez González *et al.* (1995) described a new PCR test system for the amplification of *M. bovis* and *M. agalactiae* that was based on the 16S rRNA gene. Since this gene occurs as two copies per genome, the PCR is potentially more sensitive than PCRs for other genes, for which there is only a single copy. However, the PCRs developed failed to distinguish *M. agalactiae* and *M. bovis.* This reflected the very close relationship between the two species, which show only eight different base pairs in the 16S rRNA gene. Although it is rare to find *M. bovis* in sheep and goats or *M. agalactiae* in cattle, the possibility of mis-identification may have serious implications. At the start of the practical work for this thesis an improved PCR method was described at an EC COST meeting (Thessaloniki, April 1996) which was later published in the proceedings (Johansson *et al.*, 1996b). The aims of the work described in this chapter were to:

- independently evaluate the PCR of Johansson *et al.* (1996b) for the identification of *M. bovis*
- incorporate a PCR procedure in the routine detection of *M. bovis* which would improve identification and reporting times and reduce the use of specific *M. bovis* antiserum
- develop a rapid PCR-based method that could differentiate *M. agalactiae* and *M. bovis*.

2. 2. MATERIALS AND METHODS

2. 2. 1. MYCOPLASMA STRAINS USED

The type strains of *M. agalactiae* (10123) and *M. bovis* (PG45) were obtained from the National Collection of Type Cultures, Colindale, London. The origin of all other test strains is listed in Table 2.1. Preliminary experiments to optimise the PCR and investigate 'hot start' PCR methods, used the strains detailed in section 2. 2. 5. The *M. bovis* and *M. agalactiae* PCR was further developed using DNA template from: *M. agalactiae* ALK 3056 and NVI 1; *M. bovis* 98B96, 97B96, 96B96, 70B96, 171B96, 80B96, 119B96 and NVI 2; *M. bovirhinis* 12B96, 62B96 and 126B96; and *M. bovigenitalum* 6B640. PCR was also performed on extracted

DNA, mycoplasma cells direct from culture media and cultured mycoplasma cells washed once in phosphate buffered saline (PBS), from a further 15 *M. bovis* isolates. These were: 199B96, 204B96, 198B96, 196B96, 190B96, 171B96, 160B96, 124B96, 98B96, 97B96, 96B96, 91B96, 85B96, 70B96 and 58B96. The latter isolates were all derived from pneumonic cattle during 1996; they were identified by biochemical characterisation (glucose fermentation –ve, arginine hydrolysis –ve, and formation of a film on Eaton's broth medium), followed by a film inhibition test using anti-*M. bovis* (type strain) rabbit antiserum.

TABLE 2. 1. DETAILS OF MYCOPLASMA ISOLATES USED FOR ESTABLISHING THE *M. BOVIS* PCR

STRAIN REFERENCE	IDENTIFICATION	SOURCE	PLACE OF ISOLATION
ALK 3056	*M. agalactiae*	Ovine	Turkey
98B96	*M. bovis*	Bovine lung	Longstanton, Cambridgeshire
97B96	*M. bovis*	Bovine lung	Knaresborough
96B96	*M. bovis*	Bovine nasal swab	Aberystwyth
70B96	*M. bovis*	Bovine nasal swab	Aylesbury
171B96	*M. bovis*	Bovine lung	Shrewsbury
12B96	*M. bovirhinis*	Bovine nasal swab	Thirsk
62B96	*M. bovirhinis*	Bovine nasal swab	Coventry
126B96	*M. bovirhinis*	Bovine lung	Thirsk
6B640	*M. bovigenitalum*	Bovine	Not Known
80B96	*M. bovis**	Bovine lung	Thirsk
119B96	*M. bovis**	Bovine lung	Thirsk
NVI1	*M. agalactiae*	Ovine	Sweden
NVI2	*M. bovis*	Bovine	Sweden
81B96	*M. bovis*	Bovine lung	Thirsk
82B96	*M. bovis*	Bovine lung	Thirsk
119B96	*M. bovis*	Bovine lung	Thirsk
120B96	*M. bovis*	Bovine lung	Thirsk
121B96	*M. bovis*	Bovine lung	Thirsk
58B96	*M. bovis*	Bovine	Preston
85B96	*M. bovis*	Bovine nasal swab	Preston
91B96	*M. bovis*	Bovine nasal swab	Preston
124B96	*M. bovis*	Bovine lung	Bury St Edmunds
160B96	*M. bovis*	Bovine lung	Aberystwyth
171B96	*M. bovis*	Bovine lung	Shrewsbury
190B96	*M. bovis*	Bovine lung	Thirsk
196B96	*M. bovis*	Bovine nasal	Bury St Edmunds
198B96	*M. bovis*	Bovine lung	Thirsk
199B96	*M. bovis*	Bovine lung	Thirsk
204B96	*M. bovis*	Bovine lung	Shrewsbury

* Atypical *M. bovis* isolates see section 2.1.2.

2. 2. 2. MYCOPLASMA GROWTH

All mycoplasmas were cultured in Eaton's broth medium (Nicholas and Baker, 1998) at 37°C in 5 % carbon dioxide in air for 24 hours to give an approximate cell concentration of 5×10^8 cfu per ml. Eaton's broth medium was comprised of (l^{-1}): Difco PPLO broth 21.0 g; Difco yeast extract, 10.0 g; glucose 10.0 g; DNA, 0.02 g; uninactivated horse serum, 200 cm^3; polymixin B (100,000 IU per cm^3 solution), 5.0 cm^3; ampicillin, 100 mg in 1.0 cm^3; phenol red indicator (0.2% solution) 12.5 cm^3; distilled water, approximately 780 cm^3. For primary isolation, the following bacterial inhibitors were also added: thallium acetate 0.05 g; penicillin 120 mg; and sulphadimidine 0.05 g. The pH of the medium was adjusted to pH 7.6 to 7.8 using 1M NaOH and sterilised by passing through a 0.2 µm filter.

2. 2. 3. DNA EXTRACTION

DNA was extracted from 1.5 ml of a 24-hour broth culture using a rapid commercial DNA extraction kit according to the manufacturer's instructions (Isoquick, ORCA Research, Garden Grove, USA). The kit was based on guanadine thiocyanate extraction followed by sodium acetate/ethanol DNA precipitation. The DNA was resuspended in 50 µl of sterile high performance liquid chromatography (HPLC) grade water prepared at VLA by passing distilled water through a Millipore ultrapure filtration unit. The presence of DNA was confirmed by horizontal agarose gel electrophoresis as described in section 2.2.6.

2. 2. 4. TEMPLATE DNA FOR PCRs

Except where stated, template DNA for PCRs was obtained by extraction from broth culture as described in Section 2.2.3. In experiments with *M. bovis*, to improve detection times, the use of whole cells was investigated. Cells were grown for 24 hours in Eaton's broth medium and were either diluted 10^{-1} in fresh medium or resuspended in an equal volume of PBS, pH 7.2 (Oxoid) after centrifugation at 12,000 *g* for five minutes. Cells diluted (10^{-1}) in PBS were also tested. The number of cells in the broth cultures was determined by a surface-drop plate method (Postgate, 1969). The agar used was Eaton's agar with ampicillin; plates were incubated for 48 hours at 37°C in 5 % carbon dioxide in air.

2. 2. 5. PRELIMINARY EXPERIMENTS AND TIIE TESTING OF M. BOVIS PCR SPECIFICITY

Preliminary experiments were conducted using DNA (extracted as described in Section 2.2.3) from the type strains of *M. agalactiae* and *M. bovis* and *M. bovis* field strains 80B96, 81B96, 82B96, 119B96, 120B96 and 121B96 (Table 2.1). Three hot start methods were investigated:

Ampliwax beads (Perkin Elmer) were used with a PCR mix containing: 10x running buffer (2 µl), 25 mM magnesium chloride (4 µl), dNTPs (2 µl), 40 pmol primers (1 µl each) and water (10 µl). One wax tablet was added per tube. The wax was melted by heating at 70°C for 1 minute before cooling to allow the wax to set and form a barrier between the PCR mix and the sample mix that was added later. The sample mix contained: 10x running buffer (3 µl), *Taq* (0.5 µl), DNA template (1 µl) and water (25.5 µl). The hot start began when the PCR cycling temperature reached the melting point of the wax, and the two component solutions mixed.

The *Taq*Gold (PerkinElmer) hot start was used as described by the manufacturers. This hot start method is simpler than the wax beads method as it uses a single reagent mix, prepared by combining the mixes described, but with *Taq*. An additional heating step at 94°C for eight minutes was used before the *Taq*. enzyme was released and the PCR allowed to start.

The final hot start method used was a manual one. The component solutions were as described in Table 2.2. The first solution was heated at 94°C for four minutes before the second solution containing the *Taq* enzyme was pipetted into the reaction tube allowing cycling to commence.

2. 2. 6. PCR FOR M. BOVIS AND M. AGALACTIAE

Both PCR methods were conducted under the same conditions, but using different primer sequences. Details of the primers are given in Table 2.3. and the PCR mix in Table 2.2. Except in preliminary experiments (see Section 2.3.1.) a hot start was used at 94°C for 4 minutes (Johansson *et al.*, 1996b) using a Perkin Elmer thermal cycler. With every PCR a known positive DNA template was run as a positive control and a water sample as a negative control.

Except where stated the cycling conditions used were: 94°C denaturation for 45 seconds; 65°C annealing for one minute; followed by 72°C polymerisation for two minutes. This cycle was repeated 30 times before final polymerisation at 72°C for five minutes.

Amplicons were separated on a 1 % agarose gel. The PCR reaction mixture (5 µl) was mixed with 2 µl of loading buffer (40 % w/v sucrose in water containing 0.25 % w/v bromophenol blue) and loaded onto the gel which was electrophoresed at 110 volts for 30 minutes. The gels were stained with ethidium bromide and the DNA products were visualised by ultraviolet transillumination (Maniatis *et al.*, 1982).

2. 2. 7. RESTRICTION ENZYME ANALYSIS FOR DIFFERENTIATING M. BOVIS AND M. AGALACTIAE

The PCR product (8 µl PCR reaction mixture) was digested at 37°C for 2 hours with 3 µl (3 units) of restriction enzyme, *Sfc*I (New England Biolabs, UK) after addition of 2 µl 10× concentrated buffer (as supplied with the enzyme by New England Biolabs, UK) and 7 µl HPLC grade water. The digested products were separated on a 1.5 % agarose gel and visualised as described in Section 2.2.6. The recognition site for *Sfc*I is 5'-...CTPuPyAG...-3' and it cuts between the cytosine and thymine.

TABLE 2. 2. REAGENTS USED IN THE *M. AGALACTIAE* AND *M. BOVIS* PCR's

REAGENT	**VOLUME**
HPLC grade H_2O	28 µl
Buffer 4 (Perkin-Elmer): 250 mM Tris-HCl buffer (pH 8.3), 500 mM KCl, 20 mM $MgCl_2$, 1% Tween.	5 µl
dNTPs (Pharmacia ultrapure), 2 mM	5 µl
Magnesium chloride (Perkin-Elmer) 25 mM	4 µl
Primer 1 at 40 *p*mol/µl	0.5 µl
Primer 2 at 40 *p*mol/µl	0.5 µl
Mineral oil	50 µl
DNA template	5 µl
After heating at 95°C for 4 minutes:-	
AmpliTaq (PerkinElmer) 5U/µl	0.2 µl
Buffer 4 (PerkinElmer) as above.	0.2 µl
HPLC grade H_2O	1.6 µl
Total reaction volume 50 µl with 50 µl of mineral oil to prevent evaporation.	

TABLE 2. 3. PRIMERS USED IN THE *M. AGALACTIAE* AND *M. BOVIS* PCR's

PRIMER NAME	PRIMER SEQUENCE
M. agalactiae Forward	5'-GAAGAAAAAGTAGC**G**TAGGAAATGA**C**-3'
M. bovis Forward	5'-GAAGAAAAAGTAGC**A**TAGGAAATGA**T**-3'
M. agalactiae Reverse	5'-CGTCGTCCC**T**ACCTTCCTCCC**A**-3'
M. bovis Reverse	5'-CGTCGTCCCCACCTTCCTCCC**G**-3'

Bases underlined in bold highlight the small differences in *M. agalactiae* and *M. bovis* primer design (Johansson *et al.*, 1996b).

2. 3. RESULTS

2. 3. 1. M. BOVIS PCR SPECIFICITY

The PCR developed was based on the primers of Johansson *et al.* (1996b) for *M. bovis.* Preliminary work was carried out to establish optimal conditions for the PCR and to investigate specificity. With a low (55°C) annealing temperature it was confirmed that optimal amplification was produced using a buffer containing 2 mM $MgCl_2$. However, the *M. bovis* PCR procedure also resulted in amplification of *M. agalactiae* DNA and the DNA of *M. bovirhinis* and *M. bovigenitalium* (Figure. 2.1.). To overcome difficulties with non-specific amplification during the PCR a 'hot-start' was incorporated into the procedure. The first method attempted was the 'wax beads' technique (See Section 2.2.5.). Using an annealing temperature of 55°C amplicons were produced by all of the cultures tested: *M. bovis* (6 strains); *M. bovirhinis* (2 strains); *M. bovigenitalium* (1 strain); and *M. agalactiae* (1 strain). These amplicons were all of the expected size of approximately 734 base pairs. When restriction enzyme analysis was performed on these amplicons *M. bovis* and *M. agalactiae* could easily be differentiated from each other. *M. bovis* gave four bands of approx; 601, 423, 178 and 133 base pairs compared to *M. agalactiae* which gave two bands at 601 and 133 base pairs. *M. bovirhinis* appeared to be almost totally digested, giving only one low weight band. However, *M. bovigenitalium* gave a profile similar to that of *M. bovis* with bands at 601, 423, 178 and 133 base pairs (See Figure 2.2).

Using the *Taq*Gold (PerkinElmer) hot start PCR method (See Section 2.2.5.) no amplicons were produced at an annealing temperature of 65°C. At an annealing temperature of 55°C amplicons of approximately 734bp were produced for both *M. agalactiae* and *M. bovis*, although two of the seven *M. bovis* strains tested in this PCR, the amplicons were very weak (See Figure 2.3.). Increasing the annealing temperature would therefore reduce the sensitivity of the PCR.

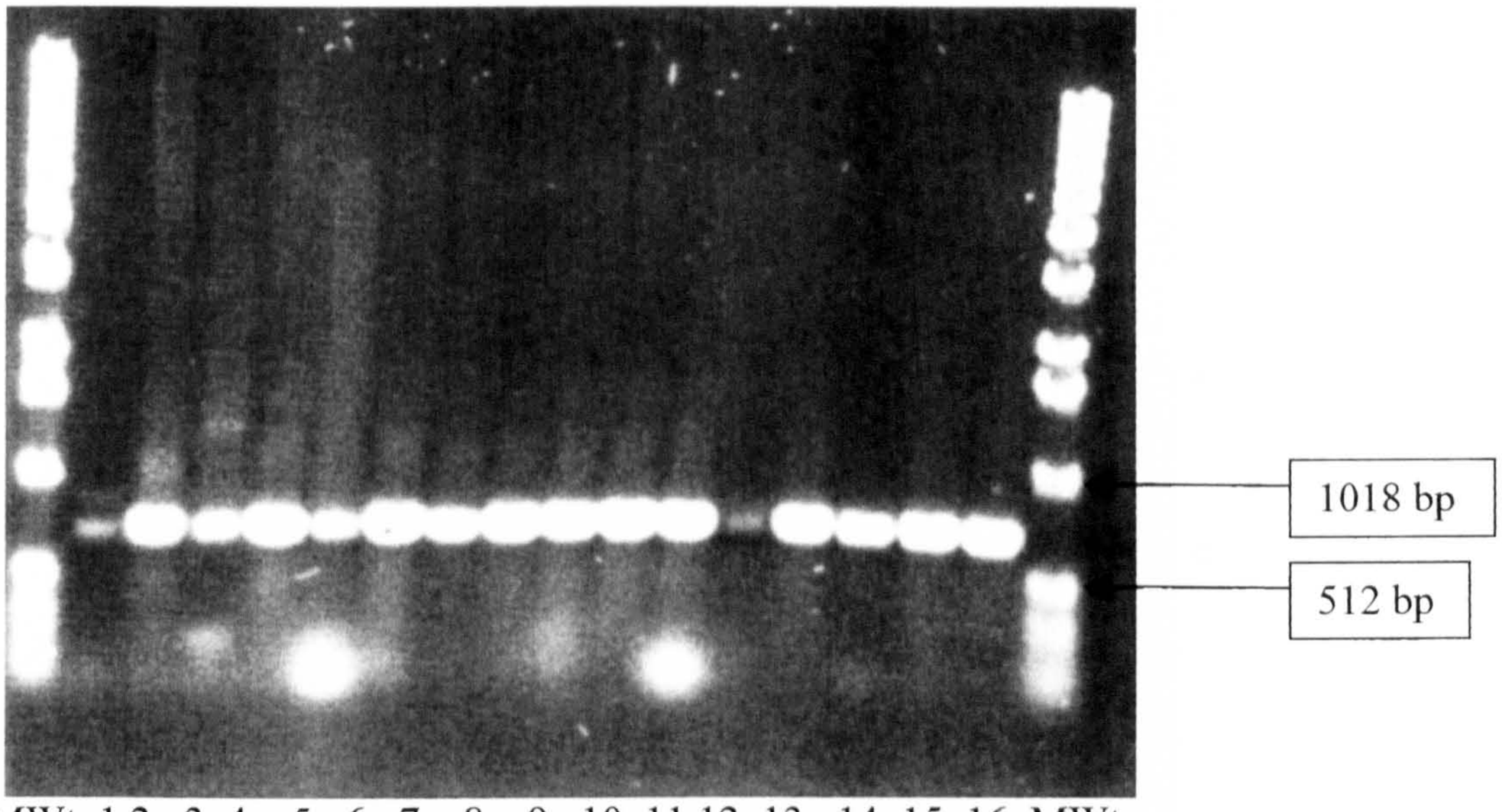
In the third hot start PCR method, the sample was heated at 94°C for four minutes before the *Taq* enzyme was added and the cycling started. Using *M. bovis* specific primers, this PCR amplified only *M. bovis* DNA at an annealing temperature of 65°C. DNA from other test strains was not amplified. At an

annealing temperature of 55°C, both *M. bovis* and *M. agalactiae* DNA were amplified, but no bands were obtained using *M. bovirhinis* and *M. bovigenitalium.*

2. 3. 1. 1. COMPARISON OF TEMPLATES FOR M. BOVIS PCR

The 15 strains of *M. bovis* analysed all produced amplicons of 734 bp from DNA templates and from cells suspended in PBS, but only 14 produced amplicons from cells suspended in growth medium; six of these amplicons were very weak. DNA resuspended in growth medium produced amplicons in all samples. Details are given in Table 2.4. All of the cultures tested gave plate counts in the range 7×10^8 to 1×10^{10} cfu. These results clearly show, even when using DNA that there is a need to test more than one dilution of sample. In six of the DNA samples only one of the dilutions from that sample was positive, although the samples that produced amplicons showed intense staining indicating more product had been produced than from the cells either in PBS or medium. For cells in PBS only three of the sample dilutions were negative on PCR, compared with six in medium. The amplicons produced from cells in medium showed only weak banding compared to that from DNA. Only one dilution was negative in all tests, and three were negative in both the PCRs performed using cells in medium and extracted DNA.

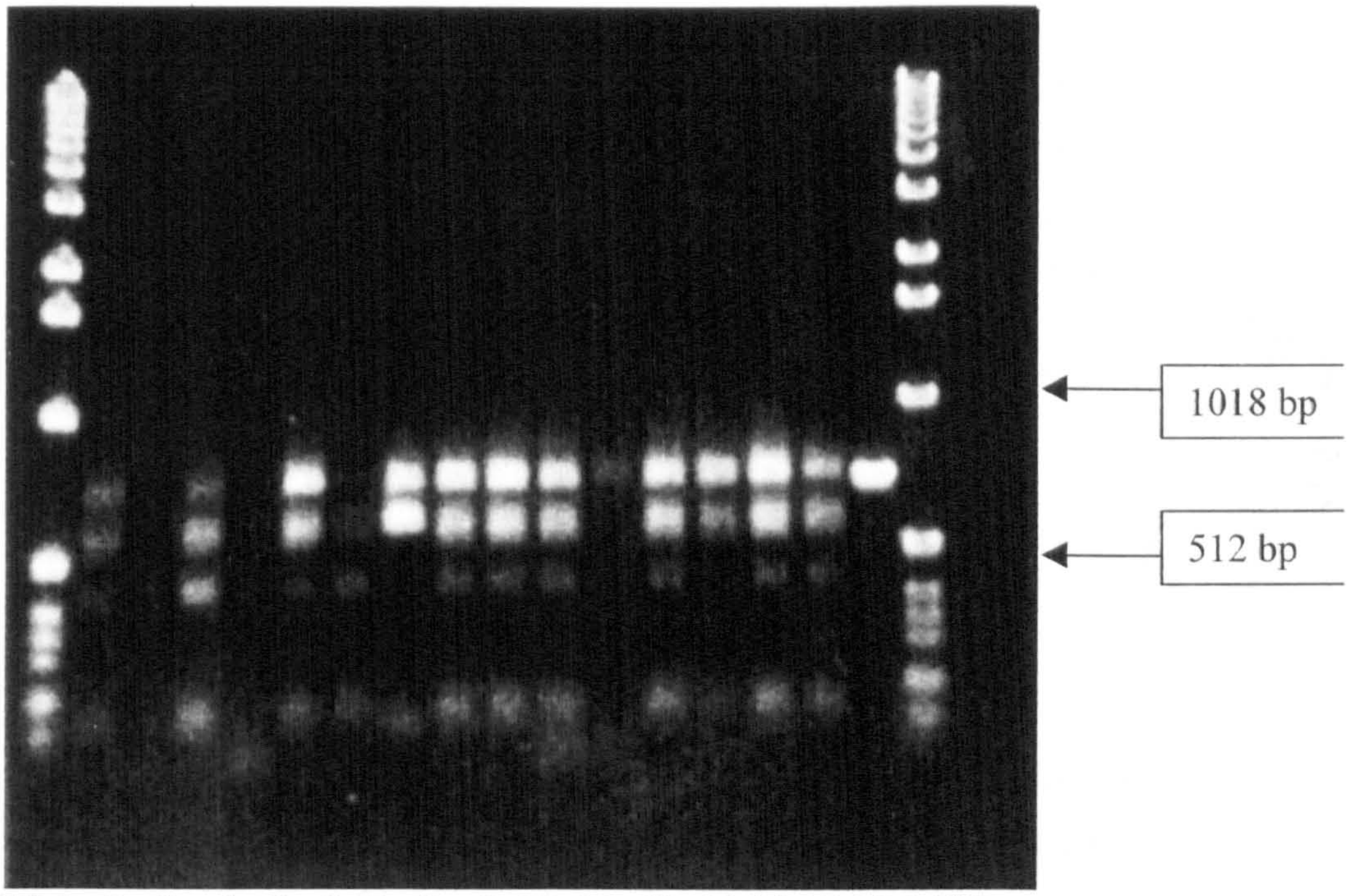
FIGURE 2. 1. PCR RESULTS USING A WAX BEAD HOT START AND A 55°C ANNEALING TEMPERATURE.



Lanes 1, 2, 4, 6, 9 to 16	shows *M. bovis* PCR amplicons,
Lanes 3 and 5	shows amplicons from *M. bovirhinis*,
Lane 7	shows an amplicon from *M. bovigenitalium*,
Lane 8	shows an amplicon from *M. agalactiae*.

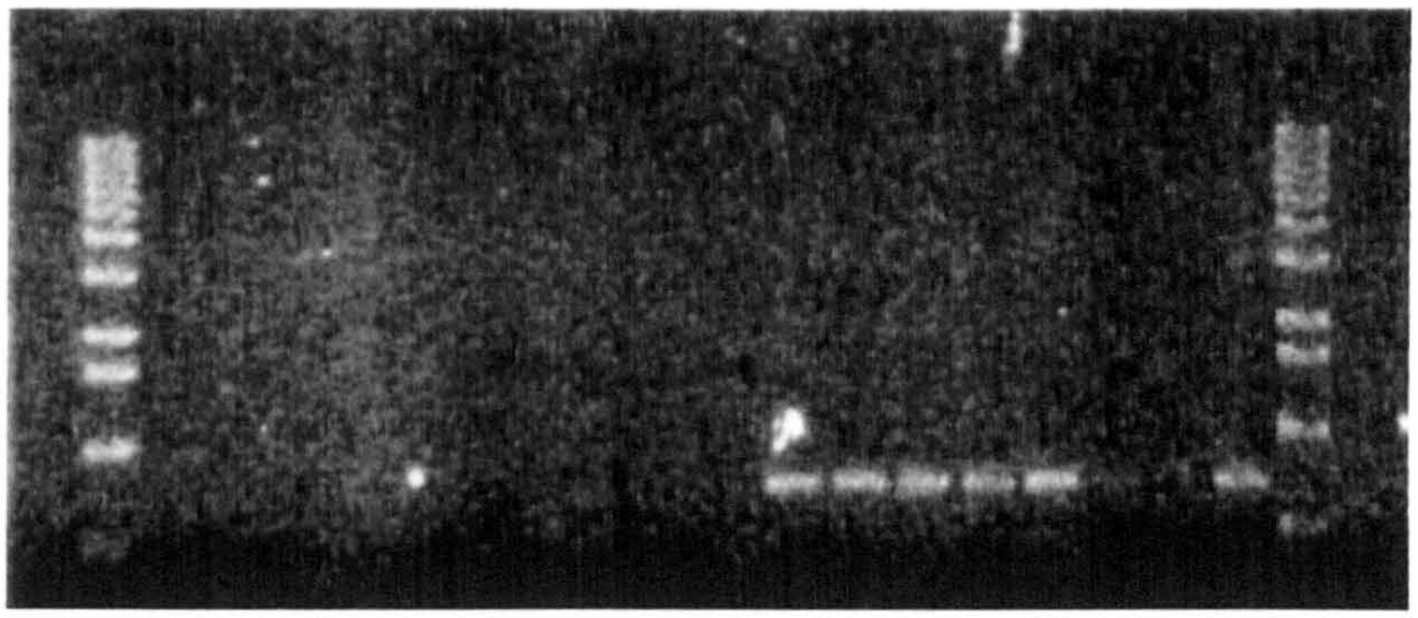
The weaker bands shown in Lanes 1 and 12 are both from the atypical *M. bovis* strain 119B96

FIGURE 2. 2. <u>Sfc</u>I RESTRICTION ENZYME ANALYSIS OF AMPLICONS FROM WAX BEAD HOT START AND A 55°C ANNEALING TEMPERATURE.



Lane 16	Uncut PCR amplicon
Lanes 1, 3, 5, 8, 9, 10 12, 13, 14, 15	Similar profiles, all from *M. bovis*
Lane 6	*M. bovigenitalium*, a weak profile similar to that from *M. bovis.*
Lane 7	*M. agalactiae*, lacks bands at approx 423 and 178 base pairs.
Lanes 2 and 4.	show no distinguishable bands, from *M. bovirhinis*
Lane 11	undigested product only, from atypical *M. bovis* isolate. 119B96

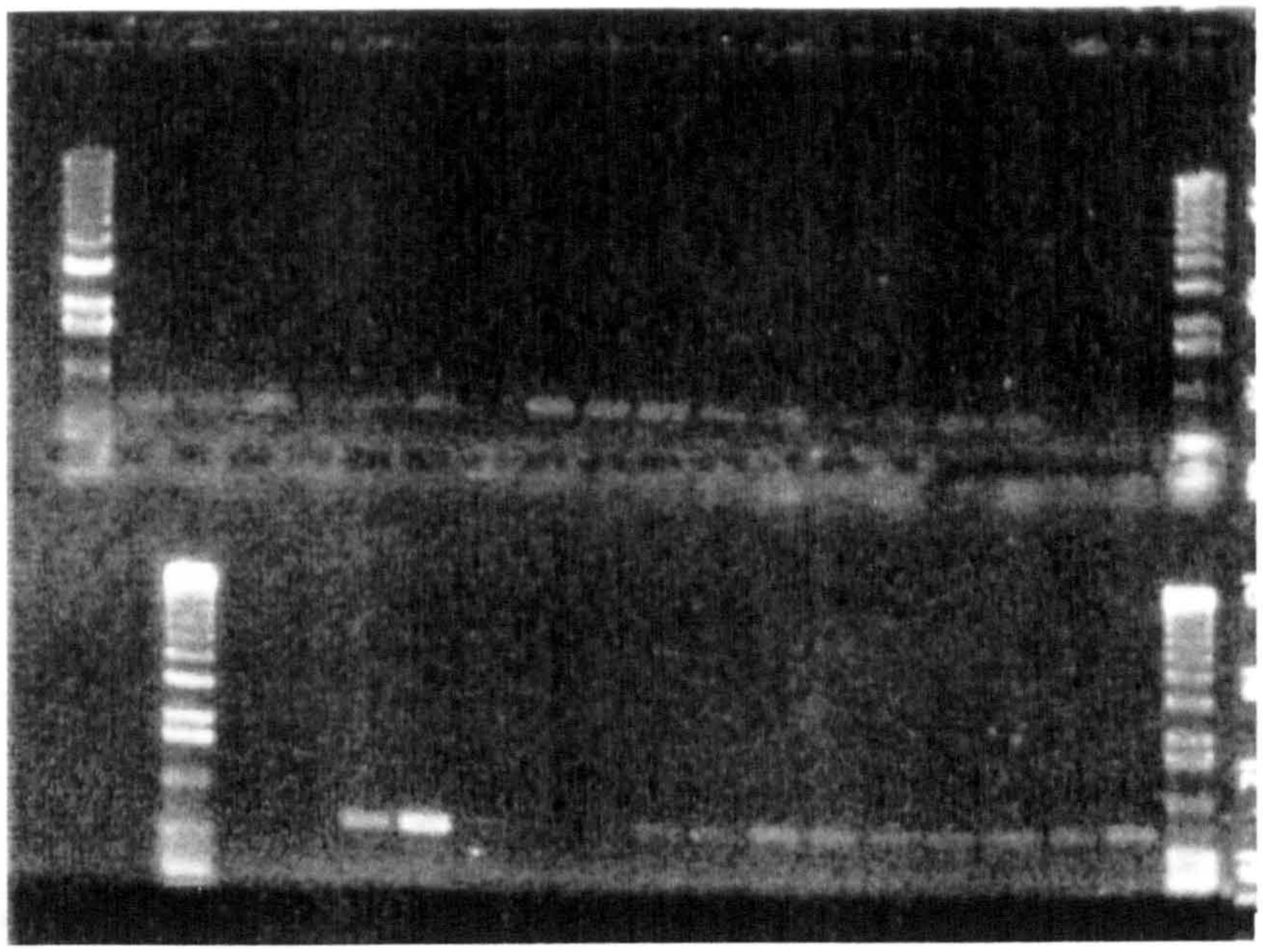
FIGURE 2. 3 PCR USING TAQGOLD HOT START



Lanes 1 to 9	No amplicons, PCR annealing temperature, 65°C
Lane 10	Negative control, PCR annealing temperature, 55°C
Lanes 11 to 17	*M. bovis* amplicons. Very weak amplicons were obtained with the *M. bovis* type strain lane 17, and atypical strain 80B96 in lane 16*.
Lane 18	*M. agalactiae* type strain amplicon.

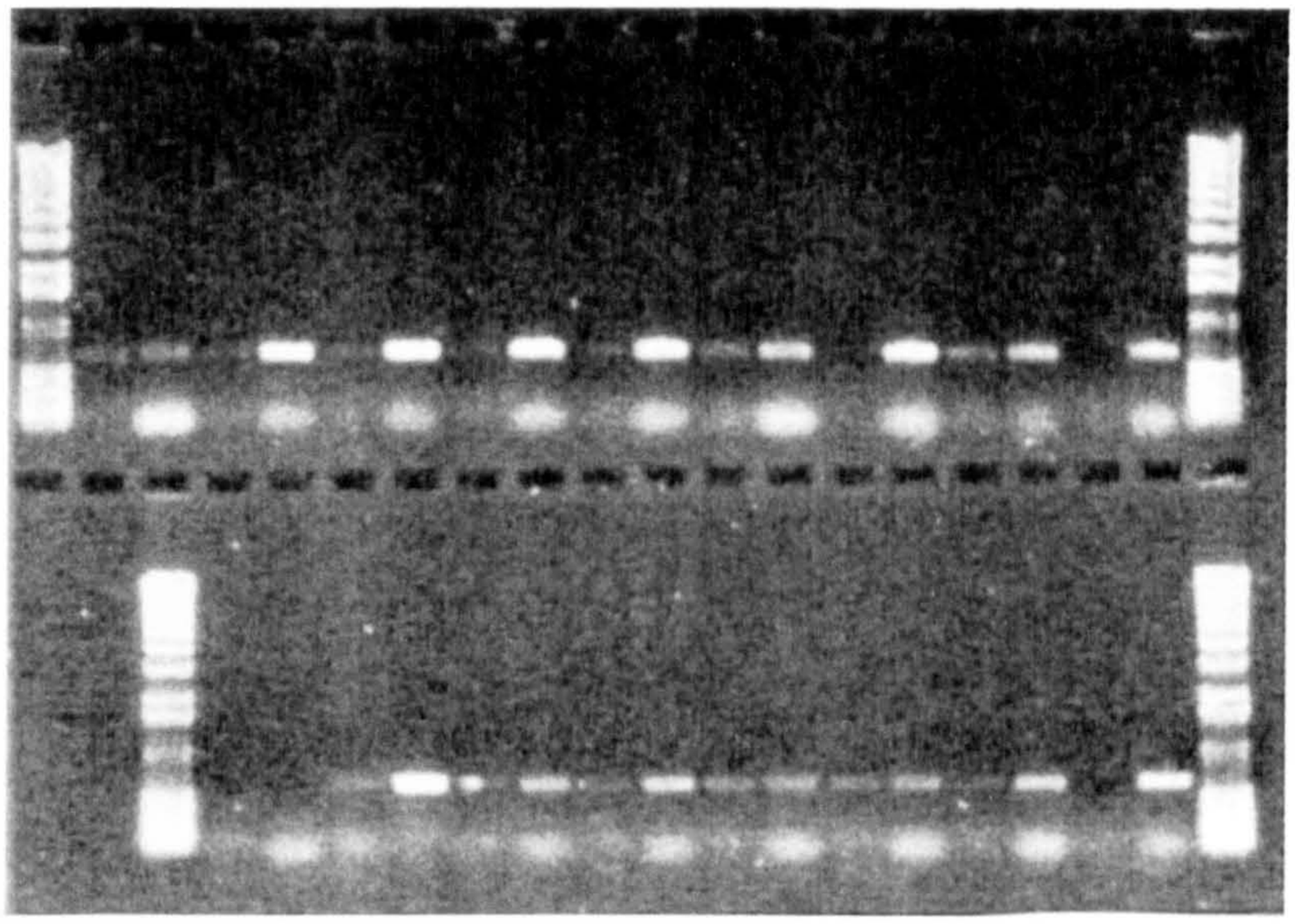
*It is worth noting that the other atypical *M. bovis* strain 119B96 gave a strong amplicon in lane 13.

FIGURE 2. 4. PCR AMPLICONS FROM CELLS IN EATON'S CULTURE MEDIA



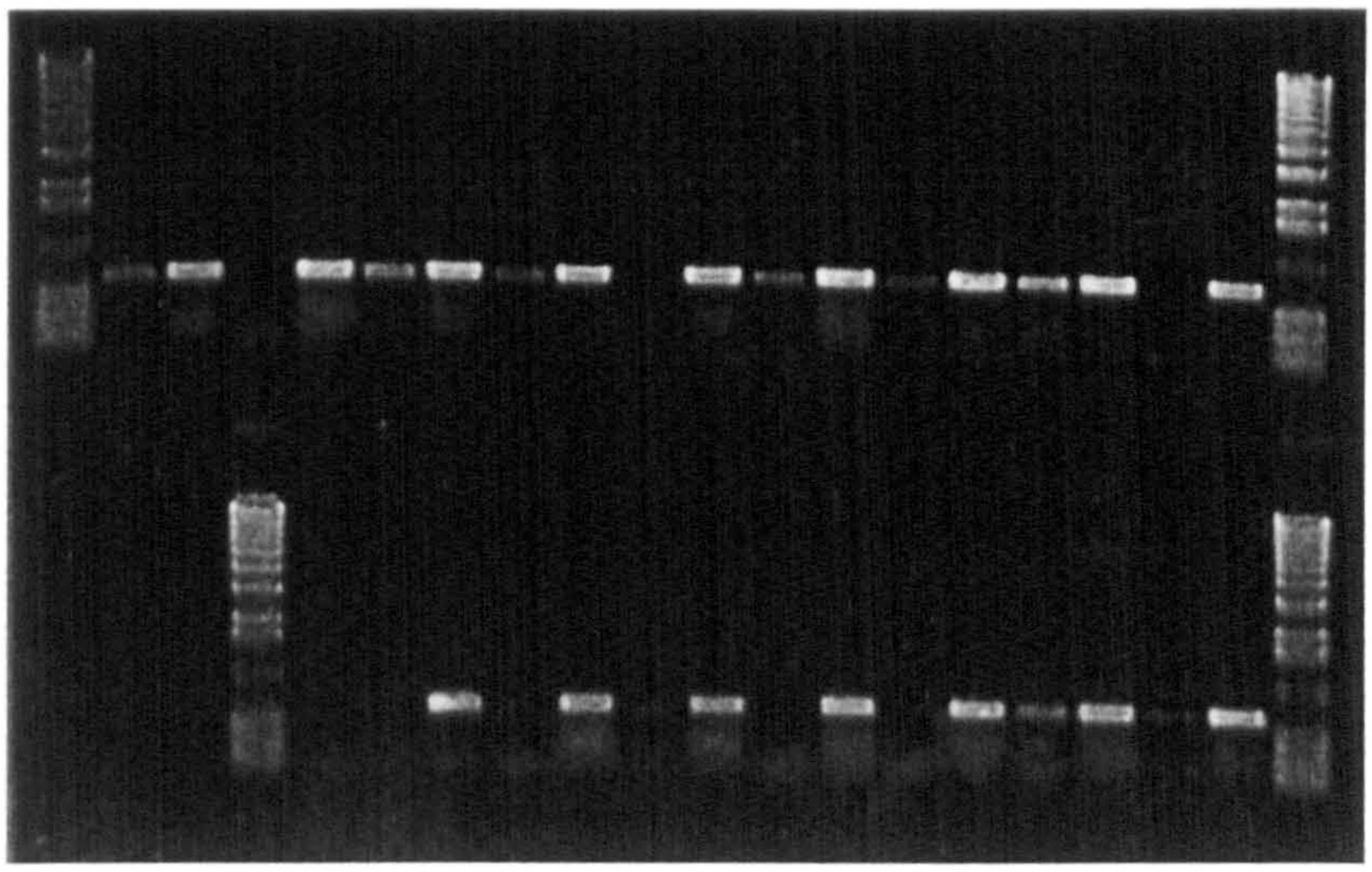
Details of isolates and interpretation of amplicons are given in Table 2.4.

FIGURE 2. 5. PCR AMPLICONS FROM CELLS WASHED IN PBS



Details of isolates and interpretation of amplicons are given in Table 2.4.

FIGURE 2. 6. PCR AMPLICONS FROM DNA EXTRACTS



Details of isolates and interpretation of amplicons are given in Table 2.4.

TABLE 2. 4. COMPARISON OF PCR PRODUCTS USING DIFFERENT TEMPLATES

SAMPLE / DILUTION		EATON'S MEDIUM-CELLS (Figure 2.4)	WASHED CELLS (Figure 2.5)	DNA EXTRACTS (Figure 2.6)
199B96	Neat	-	+++	++++
	-1	-	-	-
204B96	Neat	+	+++	++++
	-1	+	++	+++
198B96	Neat	Weak +	+++	++++
	-1	Weak +	-	++
196B96	Neat	+	++	++++
	-1	+	+	++
190B96	Neat	+	+++	++++
	-1	+	+	+
171B96	Neat	+	+++	++++
	-1	-	+	++
160B96	Neat	+	+++	++++
	-1	Weak +	+	+++
124B96	Neat	-	+++	++++
	-1	+	+	-
98B96	Neat	Weak +	++	++++
	-1	Weak +	++	++
97B96	Neat	++	+++	++++
	-1	+	-	+
96B96	Neat	Weak +	++	++++
	-1	Weak +	+	++
91B96	Neat	Weak +	++	++++
	-1	Weak +	++	-
85B96	Neat	++	++	++++
	-1	Weak +	++	-
70B96	Neat	Weak +	+++	++++
	-1	-	+	-
58B96	Neat	Weak +	+++	++++
	-1	-	+	-
+ve DNA control		++++	++++	++++
+ve DNA control		+++	++	++
-ve control		Eaton's medium -	PBS -	Not done
-ve control water		-	-	-

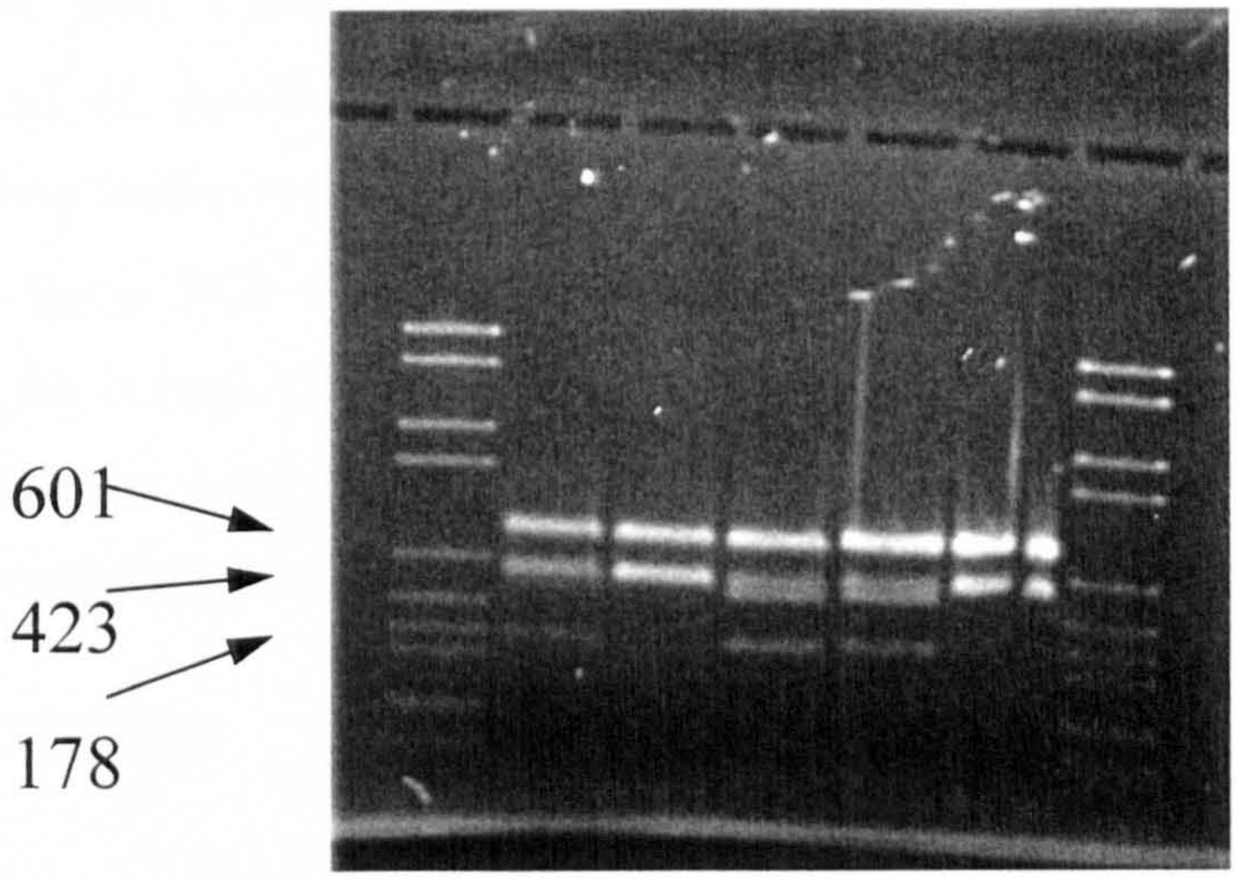
+ = Positive, an amplicon band is visible, increasing to ++++ intense band,

- = Negative.

2. 3. 2. RESTRICTION ENZYME ANALYSIS OF M. BOVIS PCR AMPLICON

Amplicons produced from either the *M. agalactiae* or *M. bovis* PCR when using a low 55°C annealing temperature can be differentiated by restriction enzyme analysis. Using the restriction enzyme *Sfc*I gives 2 distinct bands of 601 and 133bp with *M. agalactiae* see Figure 2. 7. *M. bovis* is clearly distinguished, giving 4 bands at 601, 423, 178 and 133bp. The number of bands is as expected because there is one common restriction site for *Sfc*I and another site which is unique to *M. bovis* (Johansson *et al.*, 1996b).

FIGURE 2. 7. RESTRICTION ENZYME ANALYSIS USING <u>Sfc</u>I ON PCR AMPLICONS



Lanes: 1: MWt, 2: *M. bovis*, 3: *M. agalactiae* 4: *M. bovis,* 5: *M. bovis 6: M. agalactiae* 7: MWt.

2. 3. 3. VALIDATION RESULTS

Between June 1996 and December 1999, 216 bovine mycoplasma cultures submitted for routine mycoplasma identification were tested using this PCR method. From these, 136 were positive using the PCR. All of the isolates that were positive using this PCR also proved to be *M. bovis* by GIT. Of the 83 PCR negative results none were identified as *M. bovis* in culture. These were subsequently identified as; *M. bovirhinis* (44), *M. dispar* (3), *M. canis* (17), *M. arginini* (5), *Acholeplasma laidlawii* (6), *A. modicum* (1), and seven were non-viable.

Some PCR tests on non-incubated culture submissions were attempted as soon as the cultures arrived at VLA (Weybridge). These were not successful as lack of sufficient cells gave false negative PCR results, which later proved PCR positive on re-testing, when cells were clearly present in the PCR template.

2. 4. DISCUSSION

2. 4. 1. DISCUSSION: M. BOVIS PCR

From the results obtained in this study more samples were positive from the PBS treated group, than from DNA and direct from cells in medium. The template concentration may be a factor affecting this PCR. It is apparent that assay reliability using cells in growth medium can be affected by media components possibly due to the pH changes that have occurred as a result of mycoplasma growth or the presence of other inhibitors. Interestingly, Gibb and Wong (1998) showed that PCR could be inhibited by agar from bacteriological transport media. In addition, it is good practice within laboratories to use two dilutions, for example neat and 1/10 or 1/50, of the DNA or cell template for PCR as it is known that testing one concentration may give a false negative result. This phenomenon has not been explained, but may be due to excess template blocking the reaction or optimum conditions not being realised. Decreasing the annealing temperature to 55°C, thus increasing the sensitivity of the test, but reducing the specificity may have increased the number of amplicons produced, as would using more sample dilutions. However, for the routine laboratory identification of *M. bovis* the more specific annealing temperature of 65°C was used. The lower annealing temperature of 55°C

has been used without success on several occasions in an attempt to detect *M. bovis* in field samples including nasal swabs and milk, where *M. bovis* has been suspected as causing disease. Those samples have also proved to be negative on culture, on one occasion *M. dispar* was subsequently isolated from 4 of the 20 swabs examined.

Since this development work was done, the test has been carried out in parallel on more than 200 samples submitted to VLA for mycoplasma identification. All positive PCR results have been confirmed using GIT. However, this is a bit surprising for two reasons. First, it would be expected that *M. bovis* could be present in cultures where other mycoplasmas such as *M. bovirhinis* are growing rapidly, possibly outgrowing the *M. bovis* which would not then be detected by conventional culture methods, and second, that more atypical *M. bovis* isolates like those described in Section 2.1.2. would be isolated. The first may be explained, because the cultures submitted to VLA (Weybridge) have usually been sub-cultured more than once, and the second may be a rare event.

Before establishing the exact PCR methodology, preliminary tests demonstrated that the use of wax beads for the hot start was not effective as the wax melts before reaching 94°C. The recently marketed *Taq*Gold may be a suitable alternative method to providing a hot start, but the test needs re-evaluating for optimum temperature and specificity, before it could be used for routine testing. However, as *Taq*Gold only weakly amplified two *M. bovis* isolates, but gave a intense amplicon for *M. agalactiae* it may not be suitable for use in this PCR system. This work has shown that the composition of the buffer, use of dilutions of the template and the correct hot start are essential. The conditions used for the *M. bovis* PCR are therefore critical for correct performance of the test.

Restriction enzyme analysis of the PCR product was not considered necessary as the *M. bovis* was shown to be specific at high stringency, and it is unlikely that *M. agalactiae* will be isolated from cattle, although this cannot be totally discounted. In 1999 an isolate from a goat farm in Kent was submitted to VLA (Weybridge). On culture it showed the same growth characteristics as *M. agalactiae.* This PCR with *SfcI* restriction enzyme analysis quickly confirmed that this isolate was in fact *M. bovis.* This demonstrates the use of this PCR, which gave rapid results and avoided the need for implementing exotic disease control measures.

Since completing this work other PCR systems have been developed for the species identification of *M. bovis* and *M. agalactiae* (Dedieu *et al.*, 1995; Tola *et al.*, 1996; Subramaniam *et al.*, 1998). Subramaniam *et al.*'s (1998) PCR test is based on a different gene, the *uvr*C DNA repair gene, that has significant differences between the two species compared with only an eight base difference in the 16S rRNA gene. In a recent EU interlaboratory trial, all the PCR methods were effective but the method based on the *uvr*C gene showed clearer differentiation between *M. bovis* and *M. agalactiae* (EC COST meeting, Budapest 1998).

The *M. bovis* species specific PCR is relatively rapid in comparison with culture followed by serological identification methods which may take an additional 3 days. PCR however, does have some limitations, as culture work is still part of a daily routine for most mycoplasma samples, whereas PCR is still regarded as a specialised technique, requiring different skills and time allocation. As more PCRs methods for identifying mycoplasma species are developed the emphasis of diagnostic work will change, possibly moving towards detection from clinical specimens. Already PCR methods for *M. conjunctivae, M. canis* and *M. hyopneumoniae* are used in the mycoplasma section at VLA (Weybridge). However, with 102 mycoplasma species now described it would be inefficient to have a PCR for each species.

One recent advance is the use of 16S rRNA gene sequencing to identify mycoplasma species. This technique was used at the start of this project on two mycoplasma isolates that could not be identified, one from a peregrine falcon and one from an ostrich (see Appendix 1). Since then methods have advanced and cycle sequencing is now used with big dye labelling methods to produce larger sequence lengths from one reaction. This method has been used many times at VLA (Weybridge) to identify unusual mycoplasma isolates. This includes *M. fermentans* from a sheep and *M. phocacerabrale* from a seal. Of more relevence to this thesis is the identification and characterisation of isolates from sheep which were initially shown to be *Mycoplasma species* ovine serogroup 11. However, it has now been shown, initially by 16S rRNA sequencing and also by biochemical and serological studies that these are in fact the same species as the cattle mycoplasma *M. bovigenitalium.* Since 1995 *M. canis* has increasingly been isolated from cattle in

the UK. However, from sequencing information it is now believed that a serological cross-reaction may have mis-identified some of these isolates, as 16S rRNA sequencing shows two totally different sequences. Work on these isolates is continuing.

CHAPTER 3

3. 1. EFFICACY OF ANTIMICROBIALS AGAINST *M. BOVIS* AND *M. MYCOIDES* SUBSP. *MYCOIDES* SC

3. 1. 1. INTRODUCTION

In vitro susceptibility testing is used to identify therapeutic substances or drugs that might be clinically effective in the treatment of microbial infections. Determination of the minimal inhibitory concentration (MIC) of an antimicrobial is the established method for comparing antimicrobial susceptibility. It is defined as the highest dilution of an antimicrobial agent that inhibits the growth of a particular micro-organism in the test period (Barragry, 1994). The MIC_{50} and MIC_{90} values are respectively used to show the MIC value for 50 and 90 % of the isolates tested. The most widely used tests involve growth of the organism in broth medium or semisolid culture that is detected by turbidity or pH change. However, alternative end points may be used e.g. ATP-dependent luminometry (Bébéar and Robertson, 1996). The reported MIC data for mollicutes are highly variable, highlighting a need for improved methodology (Kenny, 1996). The specific variables include: inoculum size, incubation period, medium composition, pH and the growth phase of the inoculum. In addition, the MIC value does not necessarily reflect the cell killing (mycoplasmacidal) activity (Taylor-Robinson, 1996). This is determined by transferring cells from the MIC test into fresh medium at a sufficient dilution to render the antimicrobial ineffective. The lowest concentration where there is no subsequent growth of mycoplasma is the minimum mycoplasmacidal concentration (MMC) (Taylor-Robinson, 1996).

It is known that, because they lack cell walls, mollicutes are not susceptible to treatment by certain antimicrobials, such as the penicillins and cephalosporins that inhibit the cross-linking of amino acid chains in peptidoglycan synthesis. Mollicutes are also resistant to sulphonamides, which inhibit folic acid synthesis, and those aminoglycosides that inhibit microbial respiration (Barragry, 1994).

Mollicutes are more likely to be sensitive to other antimicrobials such as: tetracyclines, which interrupt amino acid transfer to growing peptide chains at

ribosome complexes; quinalones and fluoroquinolones, which affect DNA gyrase activity; and the macrolides and lincosamides which inhibit protein synthesis. However, the presence of resistant strains to these and other generally effective antimicrobials is well documented (Bébéar, 1996). Resistant strains include those arising from the genetic transfer of the *tetM* resistance determinant to *U. urealyticum* and *M. hominis* in the human urogenital tract (Bébéar, 1996).

The majority of reports on mycoplasma antimicrobial susceptibility have concerned the human pathogens, although some data on porcine mycoplasmas and *M. bovis* from cattle have been published (Devriese and Haesebrouck, 1991; Cooper *et al.,* 1993; Ter Laak *et al.,* 1993; Tanner *et al.,* 1993; Ball *et al.,* 1995; Martel *et al.*, 1995; Aduriz *et al.,* 1996; Katoh *et al.,* 1996; Kobayashi *et al.,* 1996; Hannan *et al.*, 1997; Pobel *et al.,* 1997). Information on the susceptibility of *Mycoplasma mycoides* subsp. *mycoides* SC is limited. Lee *et al.* (1987) investigated the sensitivity and mutation rates to antibiotic resistance in *M. mycoides* subsp. *mycoides* strain T_1 for eight antimicrobials. More recently Mazzolini *et al.* (1997) reported a study in which 16 antimicrobials were tested against *M. mycoides* subsp. *mycoides* SC (15 isolates), and also *M. alkalescens* (13 isolates) and *M. bovis* (23 isolates).

Antimicrobials are used in some mycoplasma diseases, but have not been recommended for use in the control of CBPP. They are considered ineffective and may hinder the control of the disease by suppressing the symptoms so that infected animals are not detected; these animals may then act as reservoirs of infection aiding the spread of disease (Provost *et al.*, 1987). However, new antimicrobials have been developed and a recent FAO Emergency Prevention System (EMPRES) workshop recommended that research into the role of chemotherapy be pursued (Rweyemamu and Benkirane, 1996). *M. bovis* diseases in cattle are often refractory to therapy. However, it is generally accepted that animal health measures including antimicrobial treatment must be given increased priority in disease prevention and control (Pfützner and Sachse, 1996).

In this study, the MIC and MMC values of five antimicrobials against 62 *M. bovis* and 20 *M. mycoides* subsp. *mycoides* SC isolates were determined. The *M. bovis* isolates were all from recent cases of disease in the UK, whilst the *M.*

mycoides subsp. *mycoides* SC contained isolates from CBPP outbreaks in Italy, Portugal (see Chapter 4), Spain and Africa along with the vaccine and type strain. A 'Sensititre' microtitre plate system was used; the use of this system to determine the MIC measured by cell growth by turbidity with mollicutes has not previously been reported. Cell growth turbidity was used rather than pH indicator colour change as *M. bovis* causes only a small change in media pH (<0.5 units) during growth. Following MIC determination, mycoplasmacidal activity was also determined by a microtitre plate method based on culture turbidity. A short study to determine whether antimicrobial treatment would be likely to lead to the rapid selection of resistant mutants was also undertaken. The antimicrobials selected for testing were danofloxacin mesylate, oxytetracycline dihydrate, spectinomycin dihydrochloride, florfenicol and tilmicosin. Danofloxacin mesylate is marketed by Pfizer as Advocin™, a fluoroquinolone antimicrobial that is known to be active against gram positive and negative bacteria and is specifically indicated for the treatment of bovine respiratory disease (though not specifically for mycoplasmas). Oxytetracycline dihydrate, a broad-spectrum tetracycline, is also marketed by Pfizer as Terramycin, and is licensed for use in cattle, sheep, pigs, deer, chickens and turkeys. It is used to treat a large number of bacterial infections, particularly respiratory diseases as it has good distribution in the lung tissues and respiratory secretions (Barragry, 1994). It is commonly used for the treatment of infections caused by mycoplasmas, rickettsiae and chlamydiae and for diseases such as ovine footrot, pneumonia in cattle caused by *Pasteurella haemolytica* (recently renamed as *Mannheimia haemolytica*), bovine mastitis, and chronic rhinitis in pigs caused by *Haemophilus parasuis*. Spectinomycin dihydrochloride, an aminocyclitol, is marketed by Sanofi Animal Health as Spectam; it is active against a wide range of pathogens, particularly gram negative organisms and mycoplasmas. It is used in cattle, pigs, sheep, poultry, dogs and cats against respiratory and enteric infections. Florfenicol is marketed by Schering Plough as Nuflor. It is a structural analogue to thiamphenicol and is a bacteriostatic, synthetic, broad-spectrum antimicrobial effective against most gram positive and gram negative bacteria isolated from domestic animals. It is indicated for the treatment of respiratory tract infections due to *Mannheimia haemolytica* and *P. multocida* and mainly used in cattle. Tilmicosin

is marketed by Elanco as Micotil. It is manufactured semi-synthetically and is a member of the macrolide class of antimicrobials. It is indicated for treatment of young cattle for pneumonia, associated with *Mannheimia haemolytica* and *P. multocida.*

3. 2. MATERIALS AND METHODS

The antimicrobials tested were: danofloxacin mesylate, oxytetracycline dihydrate, spectinomycin dihydrochloride, florfenicol and tilmicosin. All were obtained in pure form from Accumed International (Horley, UK) who supplied them freeze-dried, in specified quantities, in the wells of 'Sensititre' microtitre plates. The quantities per well were such that after addition of medium and inoculum, antimicrobial concentration was in the range 0.008 to 128 μg/ml.

3. 2. 1. PLATE DESIGN

The 'Sensititre' plates were designed to the layout specification shown in figure 3. 1. Each antimicrobial was tested at 15 concentrations.

FIGURE 3. 1. 'SENSITITRE' PLATE DESIGN

	1	2	3	4	5	6	7	8	9	10	11	12
A	OX 128	OX 0.5	SP 128	SP 0.5	FL 128	FL 0.5	TI 128	TI 0.5	DA 128	DA 0.5		
B	64	0.25	64	0.25	64	0.25	64	0.25	64	0.25		
C	32	0.125	32	0.125	32	0.125	32	0.125	32	0.125		
D	16	0.06	16	0.06	16	0.06	16	0.06	16	0.06		
E	8	0.03	8	0.03	8	0.03	8	0.03	8	0.03		
F	4	0.015	4	0.015	4	0.015	4	0.015	4	0.015		
G	2	0.008	2	0.008	2	0.008	2	0.008	2	0.008		
H	1	0.000*	1	0.000*	1	0.000*	1	0.000*	1	0.000*		

OX = Oxytetracycline dihydrate SP = Spectinomycin dihydrochloride

FL = Florfenicol TI = Tilmicosin

DA = Danofloxacin mesylate * negative control (no antimicrobial)

Concentrations are expressed in µg/ml of antimicrobial when diluted to 200µl.

Rows 11 and 12 of plates were not used in the present study.

3. 2. 2. MEDIA

Mycoplasma bovis and *M. mycoides* subsp. *mycoides* SC were grown in Eaton's medium (see Section 2. 2. 2. for composition; Nicholas and Baker, 1998).

3. 2. 3. ISOLATES

All of the test isolates were minimally passaged (4-5 subcultures) and stored at -70°C until required. *M. bovis* isolates (62) were all from recent submissions to VLA (Weybridge) and came mainly from cattle with respiratory symptoms. The *M. mycoides* subsp. *mycoides* SC isolates were from CBPP cases in Portugal, Italy, Spain, Australia and Africa. The origin of strains is given in Table 3.1. The identity of all strains was determined or confirmed using standard mycoplasma identification techniques Nicholas and Baker (1998) and PCR methods (Bashiruddin *et al.*, 1994a) for *M. mycoides* subsp. *mycoides* SC and the method described in Chapter 2 of this thesis for *M. bovis*.

TABLE 3. 1. ORIGIN OF *M. BOVIS* AND *M. MYCOIDES* SUBSP. *MYCOIDES* (SMALL COLONY) ISOLATES

No.	Ref. No	Species	Isolate	Submitting Regional Centre
01	133B97	*M. bovis*	Lung	Thirsk
02	36B97	*M. bovis*	Lung	Thirsk
03	109B97	*M. bovis*	Lung	Bury St Edmunds
04	33B97	*M. bovis*	Lung	Bury St Edmunds
05	245B96	*M. bovis*	Lung	Shrewsbury
06	171B96	*M. bovis*	Lung	Shrewsbury
07	103B97	*M. bovis*	Lung pool	Thirsk
08	205B96	*M. bovis*	Lung	Thirsk
09	187B96	*M. bovis*	Lung	Shrewsbury
10	160B96	*M. bovis*	Lung	Aberystwyth
11	154B96	*M. bovis*	Lung	Thirsk
12	1B97	*M. bovis*	Lung	Starcross
13	72B97	*M. bovis*	Lung	Luddington
14	226B96	*M. bovis*	Pleural Fluid	Thirsk
15	124B96	*M. bovis*	Lung	Thirsk
16	15B97	*M. bovis*	Lung	Winchester
17	82B97	*M. bovis*	Lung	Bury St Edmunds
18	68B97	*M. bovis*	Joint	Luddington
19	139B97	*M. bovis*	Lung	Penrith
20	76B97	*M. bovis*	Lung	Winchester
21	127B96	*M. bovis*	Nasal swab	Aberystwyth
22	213B96	*M. bovis*	Lung	Thirsk
23	99B97	*M. bovis*	Lung	Bury St Edmunds
24	129B97	*M. bovis*	Lung	Aberystwyth
25	132B97	*M. bovis*	Lung	Luddington
26	131B97	*M. bovis*	Lung	Starcross
28	119B97	*M. bovis*	Lung	Shrewsbury
29	117B97	*M. bovis*	Lung	Preston
30	106B97	*M. bovis*	Lung	Bury St Edmunds
31	107B97	*M. bovis*	Lung	Truro
32	101B97	*M. bovis*	Pericardium/ Trachea	Bury St Edmunds
33	95B97	*M. bovis*	Lung	Winchester
34	80B97	*M. bovis*	Lung	Bury St Edmunds
35	79B97	*M. bovis*	Lung	Preston
36	78B97	*M. bovis*	Lung	Shrewsbury
37	77B97	*M. bovis*	Lung	Thirsk
38	70B97	*M. bovis*	Uterine Fluid	Penrith
39	68B97	*M. bovis*	Joint	Luddington
40	46B97	*M. bovis*	Nasal Swab	Aberystwyth
41	40B97	*M. bovis*	Lung	Luddington
42	39B97	*M. bovis*	Lung	Shrewsbury
43	27B97	*M. bovis*	Lung	Thirsk

No.	Ref. No	Species	Isolate	Submitting Regional Centre
44	21B97	*M. bovis*	Lung	Preston
45	17B97	*M. bovis*	Lung	Aberystwyth
46	96B96	*M. bovis*	Lung	Aberystwyth
47	10B97	*M. bovis*	Lung	Thirsk
48	233B96	*M. bovis*	Lung	Winchester
49	230B96	*M. bovis*	Lung	Thirsk
50	199B96	*M. bovis*	Lung	Thirsk
51	217B96	*M. bovis*	Lung	Shrewsbury
52	124B96	*M. bovis*	Lung	Thirsk
53	186B96	*M. bovis*	Lung	Preston
54	193B96	*M. bovis*	Lung	Bristol
55	204B96	*M. bovis*	Lung	Shrewsbury
56	176B96	*M. bovis*	Lung	Starcross
57	196B96	*M. bovis*	Nasal swab	Bury St Edmunds
58	214B96	*M. bovis*	Lung pool	Thirsk
60	71B96	*M. bovis*	Lung	Thirsk
61	153B97	*M. bovis*	Lung	Penrith
62	161B97	*M. bovis*	Lung	Luddington
63	6302	*M. mycoides* subsp. *mycoides* SC		Portugal
64	6526	*M. mycoides* subsp. *mycoides* SC		Portugal
65	M545/91	*M. mycoides* subsp. *mycoides* SC		Portugal
66	130/20P	*M. mycoides* subsp. *mycoides* SC		Italy
67	Afade	*M. mycoides* subsp. *mycoides* SC		Chad
68	V5	*M. mycoides* subsp. *mycoides* SC		Australia
69	$T_1$44	*M. mycoides* subsp. *mycoides* SC		Unknown Africa
70	375	*M. mycoides* subsp. *mycoides* SC		Botswana
71	Segonia	*M. mycoides* subsp. *mycoides* SC		Spain
72	Astercous	*M. mycoides* subsp. *mycoides* SC		Spain
73	57/13	*M. mycoides* subsp. *mycoides* SC		Italy
74	192	*M. mycoides* subsp. *mycoides* SC		Italy
75	400	*M. mycoides* subsp. *mycoides* SC		Portugal
76	427	*M. mycoides* subsp. *mycoides* SC		Portugal
77	197	*M. mycoides* subsp. *mycoides* SC		Italy
78	403	*M. mycoides* subsp. *mycoides* SC		Portugal
79	NCTC 10114	*M. mycoides* subsp. *mycoides* SC		NCTC Type strain
80	B103	*M. mycoides* subsp. *mycoides* SC		Portugal
81	6512	*M. mycoides* subsp. *mycoides* SC		Portugal
82	Santander	*M. mycoides* subsp. *mycoides* SC		Spain

3. 2. 4. INOCULATION AND INCUBATION OF 'SENSITITRE' PLATES

Culture of the test strains was in 3ml quantities of Eaton's medium (without phenol red) for 24 hours at 37°C, in a CO_2 incubator (5% CO_2 in air). The medium was inoculated with approximately 100 µl of frozen culture (-70°C). After growth, optical density was measured and adjusted to OD_{450} of 0.1, equivalent to approximately 1×10^8 colony forming units (cfu) per ml. Before dilution, the OD of all cultures was in the range OD_{450} 0.085 to 0.110.

To prepare 'Sensititre' plates, one hundred and ninety µl of Eaton's medium (without phenol red) were first dispensed into wells of plates and 10 µl of inoculum (OD_{450} 0.1) were then added to each well. The plates were sealed with a film plate sealer and incubated for 48 hours at 37°C.

3. 2. 5. DETERMINATION OF MIC AND MMC VALUES

'Sensititre' plates were incubated and MIC values determined following the assessment of growth (See Section 3.2.6). To determine MMC values, 10 µl of culture from each well of the 'Sensititre' plate, was transferred to the corresponding well of a further round bottom microtitre plate. These wells each contained 190 µl of medium, thus diluting the antimicrobial 1 in 20, i.e. to below the antimicrobials effective level. Plates were then incubated as described in Section 3.2.4. for 48 hours.

3. 2. 6. READING OF TESTS

To determine the presence of absence of growth, 'Sensititre' and microtitre plates were centrifuged at 800*g* for 3 minutes to concentrate cells at the bottom of the wells. Plates were then illuminated and examined using an inverted mirror.

3. 2. 7. SELECTION OF RESISTANT MUTANTS

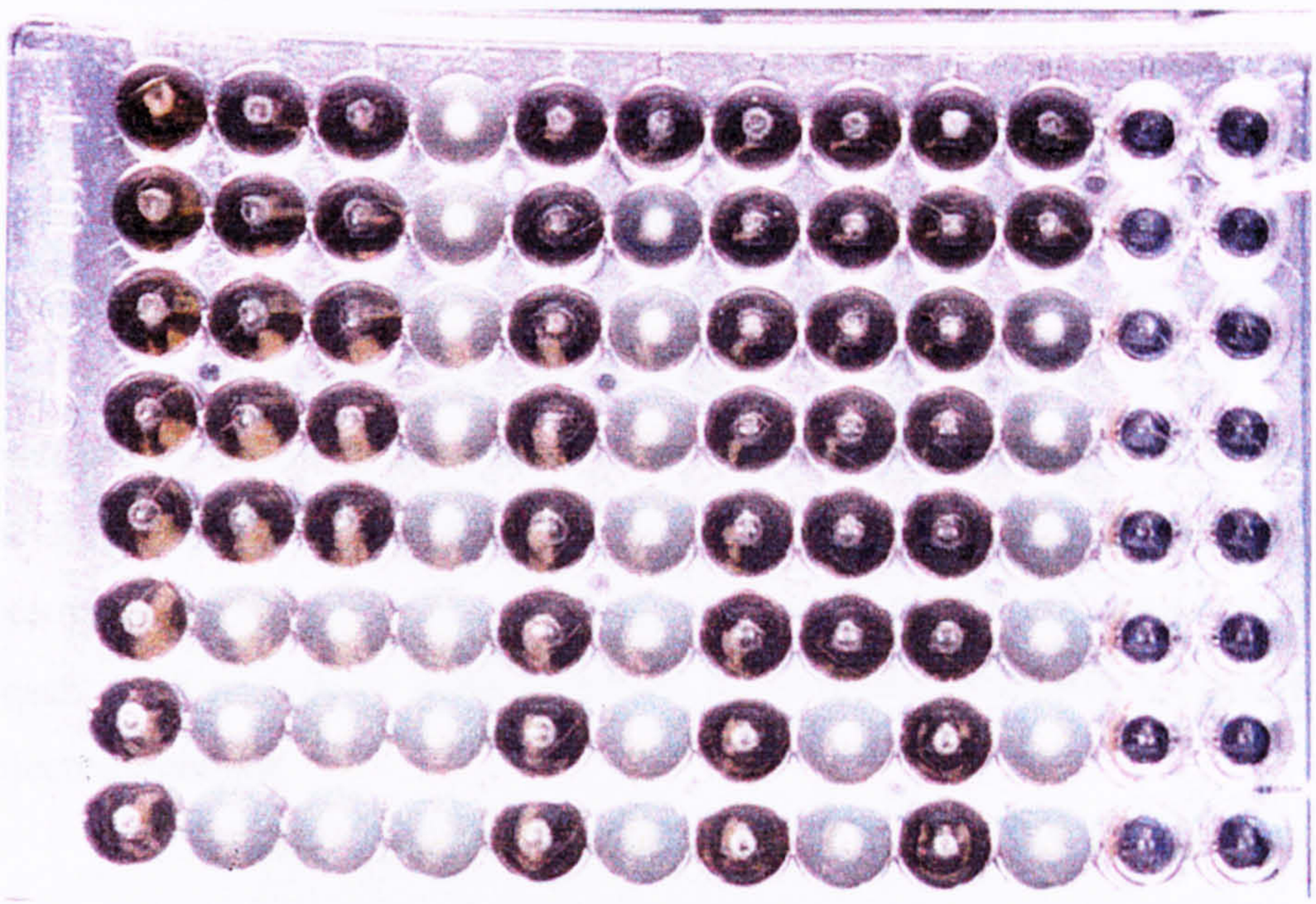
The selection of antimicrobial resistant mutants was investigated using three *M. mycoides* subsp. *mycoides* SC isolates: 400; B103 and 192 after MIC values had been determined. Fresh 'Sensititre' plates were inoculated with cells from the highest antimicrobial concentration at which growth occurred. This was repeated

for 11 successive cultures; in each subculture, the ability of cells to grow at an antimicrobial concentration above the previous MIC was determined.

3. 2. 9. DETERMINING MULTIPLE RESISTANCE

Cultures grown in 'Sensititre' plate wells containing the highest concentration of each antimicrobial were subcultured in Eaton's medium. The MIC values of these cultures for each of the antimicrobials was then determined as described in sections 3.2.4 to 3.2.6.

FIGURE 3. 2. DETERMINATION OF MIC VALUES FOR M. MYCOIDES SUBSP. MYCOIDES SC USING A 'SENSITITRE' MICROTITRE PLATE



The plate was set up as described in Section 3.2.4. using the grid from Figure 3.1. The plate shows MIC values of 0.03 μg/ml; 8.0 μg/ml; 0.5 μg/ml; 0.015 μg/ml; and 0.25 μg/ml for antimicrobials oxytetracycline dihydrate, spectinomycin dihydrochloride, florfenicol, tilmicosin and danofloxacin mesylate respectively.

3. 3. RESULTS

3. 3. 1. DEVELOPMENT OF METHODOLOGY

Antimicrobial MIC values for mollicutes are typically determined by inoculating the test strain into a series of bijou bottles containing approximately 4 ml of growth medium, with a range of antimicrobial concentrations. After inoculation, growth is determined by visual inspection of bottles for an increase in turbidity compared to uninoculated controls. In the development of methodology attempts were made to reduce preparation time and the consumption of medium and consumables by using a microtitre plate system, which has proved useful with cell-walled bacteria (Franklin and Wierup, 1982). In addition, the procedure used for the determination of growth was investigated, since mollicutes grow poorly, and the presence of growth in broth medium is sometimes difficult to determine in an objective manner.

The microtitre plate system described in Section 3.2.1. was evaluated using *M. mycoides* subsp. *mycoides* SC strain B103 and *M. bovis* strain 96B96. Using the standardised procedure in Section 3.2. With a variety of methods for determining growth, reproducible results were obtained for all of the test antimicrobials. In five experiments of this type, the MICs determined were in all cases within one doubling dilution of each other for any particular antimicrobial combination.

Detection of growth in microtitre plates using Eaton's medium without phenol red, was difficult after 24 hours, particularly for *M. bovis* strains. In many cases, it was impossible to accurately define the end point. It was easier to read plates after 48 hours, but still required some objectivity.

In Eaton's medium with phenol red, (initial pH 7.6) *M. mycoides* subsp. *mycoides* SC gave a clear colour change (red – yellow) within 24 - 48 hours, but the colour change observed with *M. bovis* cells was slight (red – orange red).

Measurement of pH confirmed that for *M. bovis*, the fall in pH during growth was less than 0.3 units. In contrast, for *M. mycoides* subsp. *mycoides* SC, the pH declined to values of <6.0. Thus, although the use of a pH change system might improve detection of growth for certain mycoplasmas it was not adopted here. However it was found that by centrifuging plates in a standard laboratory centrifuge

with a plate adapter, it was relatively easy to to determine the presence or absence of a cell pellet. When plates were observed using an inverted microtitre plate reader, MIC values could be rapidly recorded. This procedure was adopted throughout the study.

Mycoplasmacidal values are reported relatively infrequently for mycoplasmas. MMCs are often determined by incubating cultures for prolonged periods (e.g. 21 days) (Cooper *et al.*, 1993). It is assumed that the antimicrobial activity declines with incubation time, such that where growth is initially inhibited antimicrobial concentrations will fall below MIC values. This approach clearly has limitations in relation to the kinetics of antimicrobial decay, the loss of unstable medium components and the possible growth of resistant mutants, initially present in very low numbers. Thus to determine MMC values in this study, the viability of mycoplasmas exposed to antimicrobial concentrations above MIC values was determined by subculture to fresh medium. The method finally adopted was an adaptation of the microtitre system described above. Using a multichannel pipette, transfer of inocula from all the wells of incubated MIC plate, to plates containing fresh medium required only approximately one minute. After incubation, growth was detected as described above.

TABLE 3. 2. ANTIMICROBIAL MIC AND MMC VALUES OF 62 STRAINS OF *M. BOVIS*

	Minimum Inhibitory Concentration µg/ml			Mycoplasmacidal Activity µg/ml		
	Range	**MIC_{50}**	**MIC_{90}**	**Range**	**MMC_{50}**	**MMC_{90}**
Danofloxacin	0.125 – 2	0.5	0.5	0.25 – 8	0.5	1
Florfenicol	1 – 64	4	16	4 – 128	16	32
Oxytetracycline	1 – 128	32	64	2 - >128	64	128
Spectinomycin	1 – >128	4	>128	2 - >128	16	>128
Tilmicosin	4 - >128	>128	>128	16 - >128	>128	>128

TABLE 3. 3. ANTIMICROBIAL MIC AND MMC VALUES OF 20 STRAINS OF *M. MYCOIDES* SUBSP. *MYCOIDES* SC

	Minimum Inhibitory Concentrations µg/ml			Mycoplasmacidal Activity µg/ml		
	Range	**MIC_{50}**	**MIC_{90}**	**Range**	**MMC_{50}**	**MMC_{90}**
Danofloxacin	0.125 – 1	0.25	0.5	0.25 – 4	0.5	2
Florfenicol	0.25 – 8	1	2	1 – 16	8	16
Oxytetracycline	0.125 – 4	0.5	1	0.5 – 32	8	32
Spectinomycin	4 – >128	8	16	4 - >128	16	128
Tilmicosin	<0.008 – 0.25	0.015	0.03	<0.008 - >128	0.06	16

3. 3. 2. ANTIMICROBIAL RESISTANCE IN M. BOVIS

Sixty-two test strains of *M. bovis* were used. The distribution of MIC and MMC values is shown in Figure 3.3. and the data are summarised in Table 3. 2. Danofloxacin mesylate showed the lowest MIC values with values from 0.125 to 2 μg/ml; mycoplasmacidal concentrations ranged up to 8 μg/ml. Florfenicol appeared to be the next most effective antimicrobial with a range of MIC values from 1 to 64 μg/ml and a mycoplasmacidal affect at 4 to 128 μg/ml. Oxytetracycline dihydrate was effective in preventing growth at 1 to 128 μg/ml, but a mycoplasmacidal effect required greater than 128 μg/ml for two strains. Interestingly, spectinomycin showed no effect on approximately 20% of the isolates tested and tilmicosin was also ineffective for 85% of the isolates over the concentration range tested.

There was no obvious correlation of antimicrobial resistance patterns with the source of *M. bovis* isolates nor was resistance to any one antimicrobial correlated with resistance to other antimicrobials. A detailed table showing the MIC and MMC values for each individual test strain is given in Appendix 2.

3. 3. 3. ANTIMICROBIAL RESISTANCE IN M. MYCOIDES SUBSP. MYCOIDES SC

Twenty test strains of *M. mycoides* subsp. *mycoides* SC were used. The distribution of MIC and MMC values are given in Figure 3.4. and the data summarised in Table 3.3. Antimicrobial sensitivity patterns of *M. mycoides* subsp. *mycoides* SC differed from those of *M. bovis.* Danofloxacin mesylate gave the narrowest range of MIC values (0.125 to 1 μg/ml) with a mycoplasmacidal effect at 0.25 to 4 μg/ml. In most cases, tilmicosin was more effective than danofloxacin, with 90% of isolates having an MIC value of less than 0.03 μg/ml; however, three isolates had mycoplasmacidal concentrations above 16 μg/ml. Florfenicol and oxytetracycline dihydrate gave similar results with MIC values of 0.125 to 4 μg/ml and mycoplasmacidal concentrations of 0.5 to 32 μg/ml. For spectinomycin dihydrochloride, the majority (60%) of isolates had a MIC value of less than 8 μg/ml but the vaccine strain, $T_1$44 had a value in excess of 128 μg/ml. As observed for *M. bovis*, there was no clear correlation of antimicrobial resistance patterns with the

source of *M. mycoides* subsp. *mycoides* SC isolates and resistance to individual antimicrobials did not appear to correlate with resistance to other antimicrobials.

3. 3. 4. SELECTION OF ANTIMICROBIAL RESISTANT MUTANTS

To determine whether antimicrobial resistance was rapidly selected for *in vitro*, three *M. mycoides* subsp. *mycoides* SC isolates were successively subcultured 11 times in the presence of increasing antimicrobial concentrations (Table 3.4.).

Subculturing the three isolates of *M. mycoides* subsp. *mycoides* SC at the MIC level of the antimicrobial demonstrated that resistance was rapidly selected for in spectinomycin, little or no change was observed with oxytetracycline and the remaining antimicrobials showed an increase in the MIC by one or two dilutions. The results are given in Table 3.4.

The determination of cross-resistance which followed a single passage without antimicrobials before inoculating fresh 'Sensititre' plates showed that the induced resistance to spectinomycin was not lost, although the MIC value for isolate B103 was reduced to 32 μg/ml from >128 μg/ml. For the other two isolates spectinomycin MIC value remained at >128 μg/ml. No cross-resistance was observed, and remaining MIC values were no higher than two dilutions of the original MIC value (Table 3.5.), with the exception of B103 in Tilmicosin which went from 0.008 to 0.125 μg/ml. Some MIC values obtained were lower than the initial starting MIC value obtained.

TABLE 3. 4. SELECTION OF ANTIMICROBIAL RESISTANCE IN *M. MYCOIDES* SUBSP. *MYCOIDES* SC STRAINS 400, B103 AND 192

The MIC values are shown for 11 successive cultures in the presence of antimicrobials. For each subculture the inoculum used was that culture that grew at the highest test antimicrobial concentration.

	MIC VALUE (µg/ml)														
Subculture	**Oxytetracycline**			**Spectinomycin**			**Florfenicol**			**Tilmicosin**			**Danofloxacin**		
number	**400**	**B103**	**192**	**400**	**B103**	**192**	**400**	**B103**	**192**	**400**	**B103**	**192**	**400**	**B103**	**192**
0	1	0.5	1	8	4	8	1	0.5	2	0.015	0.008	0.015	0.5	0.25	0.5
1	1	1	0.5	32	16	32	8	2	2	0.03	0.03	0.06	1	0.25	0.5
2	0.25	0.125	0.25	8	16	8	1	1	0.5	0.015	0.03	0.03	0.25	0.125	0.25
3	0.125	0.125	0.125	32	16	8	2	1	1	0.06	0.03	0.015	0.5	0.5	0.5
4	0.25	0.25	0.25	32	32	8	2	2	1	0.06	0.015	0.06	0.5	0.5	0.5
5	0.125	0.25	0.25	16	128	8	2	0.5	0.125	0.03	0.015	0.015	0.5	0.5	0.5
6	0.5	0.5	0.25	64	>128	16	8	1	2	0.015	0.015	0.015	0.5	1	1
7	0.5	0.5	0.5	64	>128	>128	2	1	2	0.06	0.06	0.06	0.5	0.5	1
8	0.25	0.25	0.25	64	>128	>128	2	0.15	1	0.06	0.125	0.03	0.015	0.5	0.25
9	1	0.5	0.5	8	>128	>128	1	1	1	0.015	0.125	0.015	0.5	1	0.5
10	0.5	1	0.5	>128	>128	>128	1	1	1	0.06	0.25	0.03	0.5	1	1
11	0.5	1	0.5	>128	>128	>128	1	2	2	0.06	0.25	0.03	0.5	1	2

TABLE 3. 5. ASSESSMENT OF ANTIMICROBIAL CROSS-RESISTANCE IN *M. MYCOIDES* SUBSP. *MYCOIDES* SC STRAINS 400, B103 AND 192

The MIC values are shown in μg/ml, for the five antimicrobials from the final culture after selecting for possible antimicrobial resistance.

	Oxytetracycline			**Spectinomycin**			**Florfenicol**			**Tilmicosin**			**Danofloxacin**		
Strain	**400**	**B103**	**192**	**400**	**B103**	**192**	**400**	**B103**	**192**	**400**	**B103**	**192**	**400**	**B103**	**192**
Starting value No selection	1.0	0.5	1.0	8.0	4.0	8.0	1.0	0.5	2.0	0.015	0.008	0.015	0.5	0.25	0.5
From Oxytetracycline 11th subculture	0.125	0.125	0.5	8.0	8.0	8.0	0.5	1.0	1.0	0.06	0.03	0.03	0.5	0.25	0.5
From Spectinomycin 11th subculture	0.125	0.125	0.125	>128	32.0	>128	1.0	0.5	1.0	0.06	0.03	0.03	0.5	0.25	0.5
From Florfenicol 11th subculture	0.03	0.25	0.25	8.0	8.0	8.0	1.0	1.0	2.0	0.03	0.03	0.06	0.5	0.25	0.5
From Tilmicosin 11th subculture	0.5	0.125	0.125	8.0	8.0	8.0	1.0	0.5	1.0	0.06	0.125	0.03	1.0	0.25	0.5
From Danofloxacin 11th subculture	0.03	0.125	0.125	8.0	8.0	8.0	1.0	1.0	1.0	0.008	0.03	0.03	0.5	1.0	0.5

3. 4. DISCUSSION

Previous MIC tests carried out at VLA (Weybridge) used 4 ml volumes of medium in bijoux. That method used large volumes of medium, antimicrobials and inoculum making it expensive to perform. All dilutions and inoculations had to be performed individually, with the added inconvenience of unscrewing and replacing bijou lids. In addition the comparison of growth was again done individually either looking for swirls of growth, or pH change in comparison with the controls. Whereas the methodology used in this thesis was extremely effective and results obtained for a control *M. bovis* isolate compared favourably, within one dilution, with previous tests using broth dilutions in bottles (unpublished results). The microtitre plate method had several advantages. It was more economical with reagents, more efficient to set up than broth dilutions in bottles, as multichannel and repeating pipettes could be used. The inoculum was also standardised. Reading of results was easy, as all dilutions could be viewed at one time and did not require manipulation of bottles and shaking to observe cell growth. The MMC determination used in this study relied upon diluting the antimicrobial below its effective level to assess the killing effect of the antimicrobial, whereas Cooper *et al.* (1993) read the MIC tests after a further 14 or 21 days to give an indication of mycoplasmacidal action (See 3.3.1.). In the study by Cooper *et al.* (1993) 2 to 4 fold increases above the MIC value were reported which is similar to the results obtained in this thesis.

The MIC and MMC values obtained for *M. bovis* and *M. mycoides* subsp. *mycoides* SC isolates were similar for danofloxacin, but differed markedly for the other test antimicrobials. Oxytetracycline, spectinomycin, florfenicol and tilmicosin antimicrobials appeared relatively ineffective against *M. bovis in-vitro*, although they were more effective for *M. mycoides* subsp. *mycoides* SC, with oxytetracycline having an MIC 6 dilutions lower and tilmicosin giving the lowest MIC_{50} value obtained in this study. However, the way in which MIC values relate to the effectiveness of antimicrobials in animals is a complex issue. Antimicrobials demonstrating little or no *in vitro* activity are unlikely to be effective clinically in aiding the body's defences to eradicate infectious organisms. However, it is known that some classes of antimicrobials particularly the macrolides, may be actively

concentrated (up to 18 fold) in the phagolysosomes of cells. This may make them a more appropriate choice for chemotherapy than MIC or MMC data might suggest (Reeve-Johnson, 1999). Ter Laak *et al.* (1993) considered that mycoplasma strains were susceptible to antimicrobials *in vivo* if MIC values were equal to or less than 1 μg/ml; intermediately susceptible between 2 and 4 μg/ml; and resistant at 8 μg/ml and above. On this basis, using the MIC_{50} values, *M. bovis* is resistant to oxytetracycline and tilmicosin, intermediately susceptible to florfenicol and spectinomycin, and susceptible to danofloxacin. However, using the MIC_{90} value *M. bovis* is only susceptible to danofloxacin. For *M. mycoides* subsp. *mycoides* SC, using the MIC_{50} values, it is only resistant to spectinomycin and susceptible to the other four antimicrobials. When the MIC_{90} value is used, florfenicol moves to the intermediately susceptible category. MMC data may be more directly related to clinical effectiveness than MIC values because the MMC values relate to 'killing' rather than just growth inhibition. Classifying the antimicrobials using MMC_{90} values gives a very different picture with both *M. bovis* and *M. mycoides* subsp. *mycoides* SC being resistant to florfenicol, oxytetracycline, spectinomycin and tilmicosin. Only danofloxacin remained effective with *M. bovis* being susceptible and *M. mycoides* subsp. *mycoides* SC being intermediately susceptible.

However, most reports of MIC's use a change in pH as an indicator of growth, which presented a problem as *M. bovis* is not fermentative and only a minor pH change occurs during growth. Devriese and Haesbrouck (1991) used Tween 80 hydrolysis as an indicator of growth for *M. bovis* by looking for the presence of precipitates. However, following an initial trial using Eaton's medium with and without phenol red, it was shown (see Section 3.. 3.) that Eaton's without phenol red was suitable for both *M. bovis* and *M. mycoides* subsp. *mycoides* SC. The presence of growth was indicated by turbidity/growth precipitate in microtitre plate wells.

In this study danofloxacin mesylate was the most effective of the antimicrobials tested against *M. bovis*. Cooper *et al.* (1993) previously tested ten *M. bovis* isolates against danofloxacin mesylate and obtained a MIC_{90} of 0.25 μg/ml compared with a MIC_{90} of 0.50 μg/ml in the present study. Oxytetracycline hydrochloride was also tested in the study of Cooper *et al.* (1993) and gave an

MIC_{90} of 16 μg/ml, identical to that obtained here. Mazzolini *et al.* (1997) obtained a slightly lower MIC_{90} of 2 μg/ml for oxytetracycline against 23 strains of *M. bovis.*

Ball *et al.* (1995) tested 23 field isolates of *M. bovis* against tilmicosin and spectinomycin. They found spectinomycin gave a range of MIC values from 4 to 32 μg/ml. Mazzolini *et al.* (1997) reported a range of 4 - >256 μg/ml with an MIC_{90} of 2 μg/ml. The present results show two groups of isolates; 12 strains were resistant to >128 μg/ml whilst the remaining 50 strains were intermediately susceptible at 1 to 8 μg/ml.

Ball *et al.* (1995) found that isolates tested with tilmicosin gave MICs from <0.06μg/ml to 0.5 μg/ml or from 4 to >32 μg/ml. Two isolates in the present study had MICs of 4 μg/ml, with a further seven having MICs up to 128 μg/ml. The remaining 53 isolates were highly resistant with MICs >128 μg/ml. The isolates of Mazzolini *et al.* (1997) were similarly diverse with a range of MIC values from 0.25->32 μg/ml and a MIC_{90} of >32 μg/ml. Although the isolates tested in these three studies came from different countries (England, N. Ireland and Italy) the results all suggest that the use of tilmicosin in the treatment of *M. bovis* infections is leading to the emergence of resistant strains.

Mazzolini *et al.* (1997) obtained the following MIC values for *M. mycoides* subsp. *mycoides* SC: tilmicosin <0.03 μg/ml; spectinomycin 1 to 4 μg/ml; and oxytetracycline 0.25 to 1 μg/ml. Corresponding values in the study reported in this thesis were: <0.03 μg/ml; 4 to 32 μg/ml (with one strain >128 μg/ml); and 0.125 to 4 μg/ml, respectively. Comparisons between the studies are complicated as details of strains tested by Mazzolini *et al.* (1997) are not given. Although it is highly likely that these strains were all Italian isolates, the results are comparable to those of the present study that used strains from a wide range of countries.

One of the potential problems with the use of antimicrobials is the development of antimicrobial resistance (Chin and Marx, 1994). *In vitro* trials may be used to assess the potential for resistance-development. In this study *M. mycoides* subsp. *mycoides* SC was used to determine if resistance could be selected for by growth at antimicrobial concentrations close to the MIC level. Similar experiments were not done for *M. bovis* as many isolates were initially relatively

resistant to the test antimicrobials. The results (Table 3.4) showed that slight increases in MIC values were obtained for tilmicosin, danofloxacin, florfenicol (2 isolates) and oxytetracycline (1 isolate). These showed a trend with 1 or 2 dilution increases, but this may fall within expected levels of test variation. However, the MIC value for spectinomycin increased dramatically from 4 and 8µg/ml to greater than 128 µg/ml within 6 passages with one isolate. Interestingly, Lee *et al.* (1987) were also able to select for spectinomycin dihyrochloride resistant mutants in *M. mycoides* subsp. *mycoides* SC strain T_1. The initial MIC value for this strain was determined to be 6 µg/ml and this increased to >128 µg/ml in an apparent single step mutation. Interestingly, although Lee *et al.* (1987) used different methods, the initial MIC value of 6 µg/ml is similar to values for many *M. mycoides* subsp. *mycoides* SC isolates tested in this study. However, the strain $T_1$44 held at VLA (Weybridge) was resistant at >128 µg/ml to spectinomycin dihydrochloride suggesting that it may have acquired resistance. Resistance to spectinomycin dihydrochloride is known to develop rapidly. A mutation of the organism leading to an altered ribosome structure that no longer binds the antimicrobial would be expected to lead to high levels of resistance. This would require only a single mutation; however, it is also possible that resistant strains may have a reduced permeability towards the antimicrobial as to be able to inactivate it enzymatically.

Sub-culturing at the MIC level (Table 3.5) induced no antimicrobial cross-resistance. Interestingly, some MIC values were lower than the initial starting MIC value obtained, which was also observed in Table 3.4. This can be explained, as the inoculum for these investigations was not standardised, which emphasises the need for inoculum standardisation in MIC investigations.

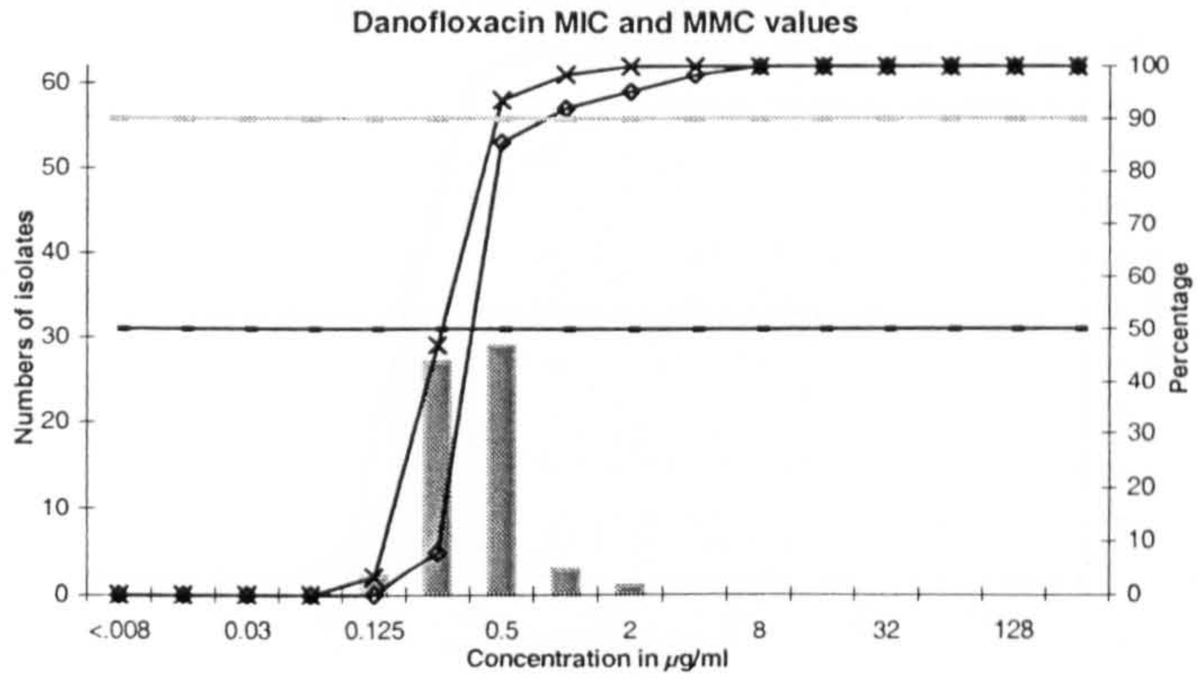
The data in this thesis appears to show greater antimicrobial resistance in *M. bovis* than in *M. mycoides* subsp. *mycoides* SC. This may be because antimicrobials are used more widely in Europe. However, in that case one might have expected to see higher MIC levels in the European *M. mycoides* subsp. *mycoides* SC isolates than in the African isolates. With the exception of the vaccine strain $T_1$44 all *M. mycoides* subsp. *mycoides* SC strains had similar MIC values with all antimicrobials. This may be explained by European control strategies for *M. mycoides* subsp. *mycoides* SC, in which infected animals are not treated, but rapidly

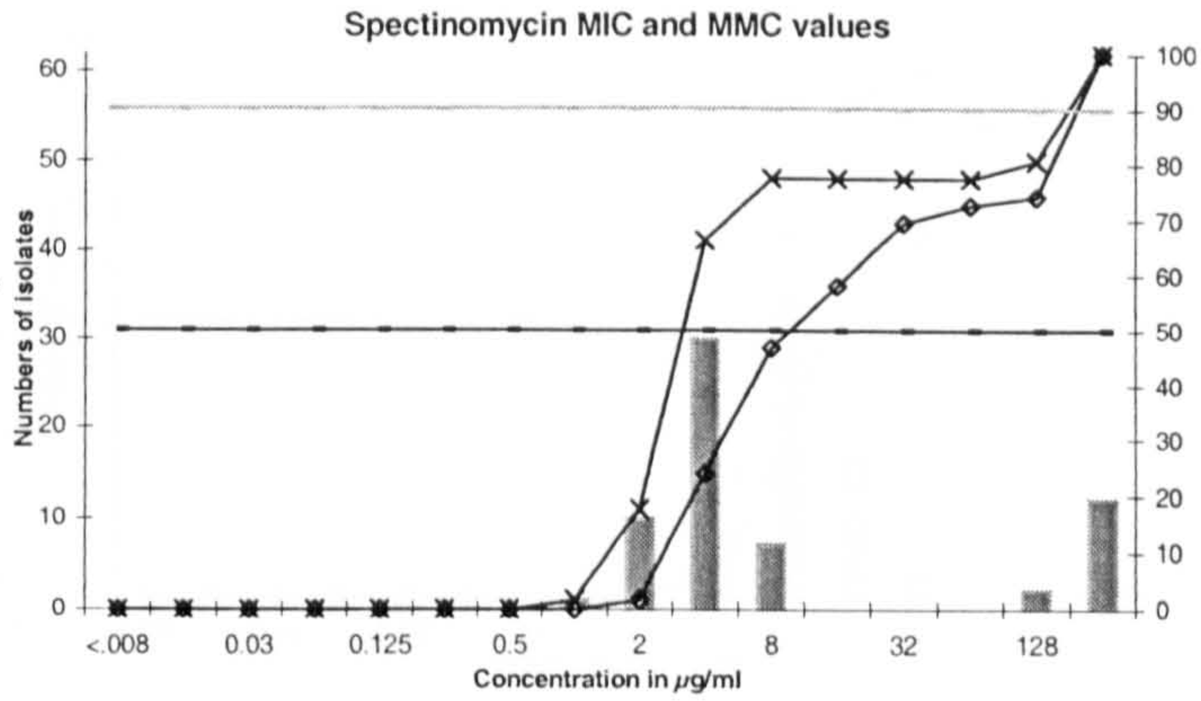
slaughtered. It would be interesting to examine isolates of *M. bovis* from African countries to determine their MIC levels.

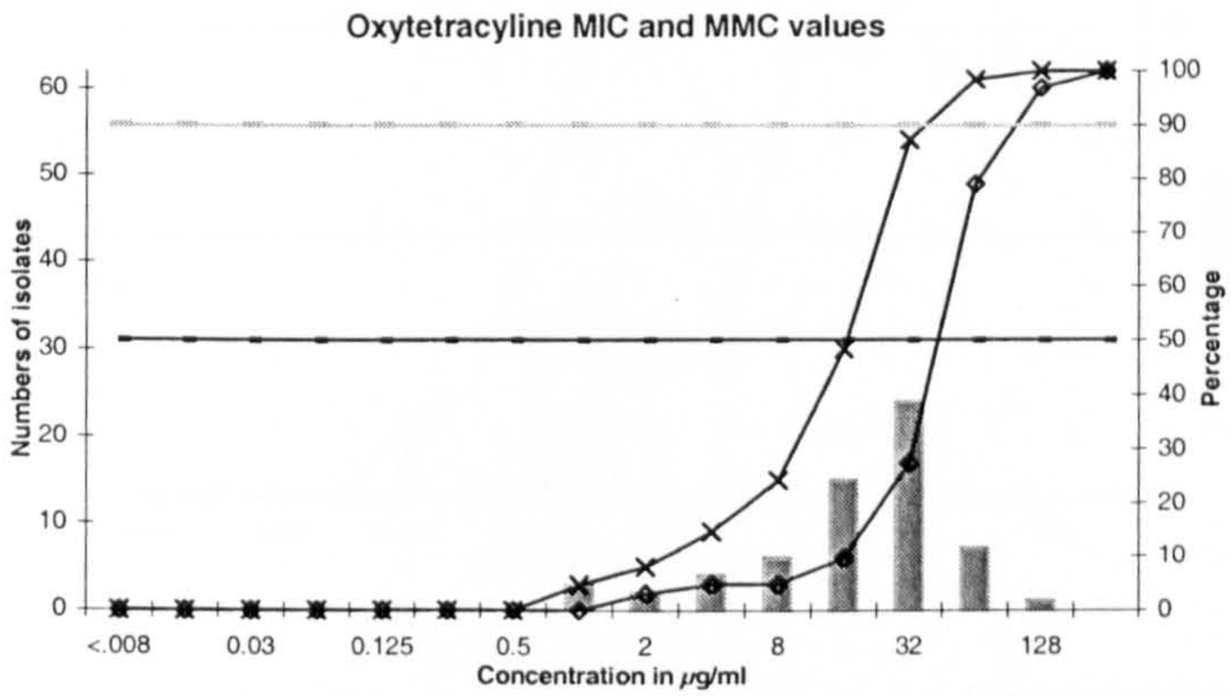
The results of the present study showed that the majority of *M. bovis* isolates have developed antimicrobial resistance. It also indicated that danofloxacin mesylate would be a useful antimicrobial to use *in vivo* against *M. mycoides* subsp. *mycoides* SC. However, both *M. mycoides* subsp. *mycoides* SC and *M. bovis* may be difficult organisms to target, because they locate in sites such as joints. The widespread use of antimicrobials would also be expected to lead to the development of resistant strains. Multiple antimicrobial resistance, particularly to fluoroquinolones, is an increasing problem in animal health, and the treatment of bacterial enteric diseases (Wray *et al.*, 1991; Nijsten *et al.*, 1993; Acar and Goldstein, 1997).

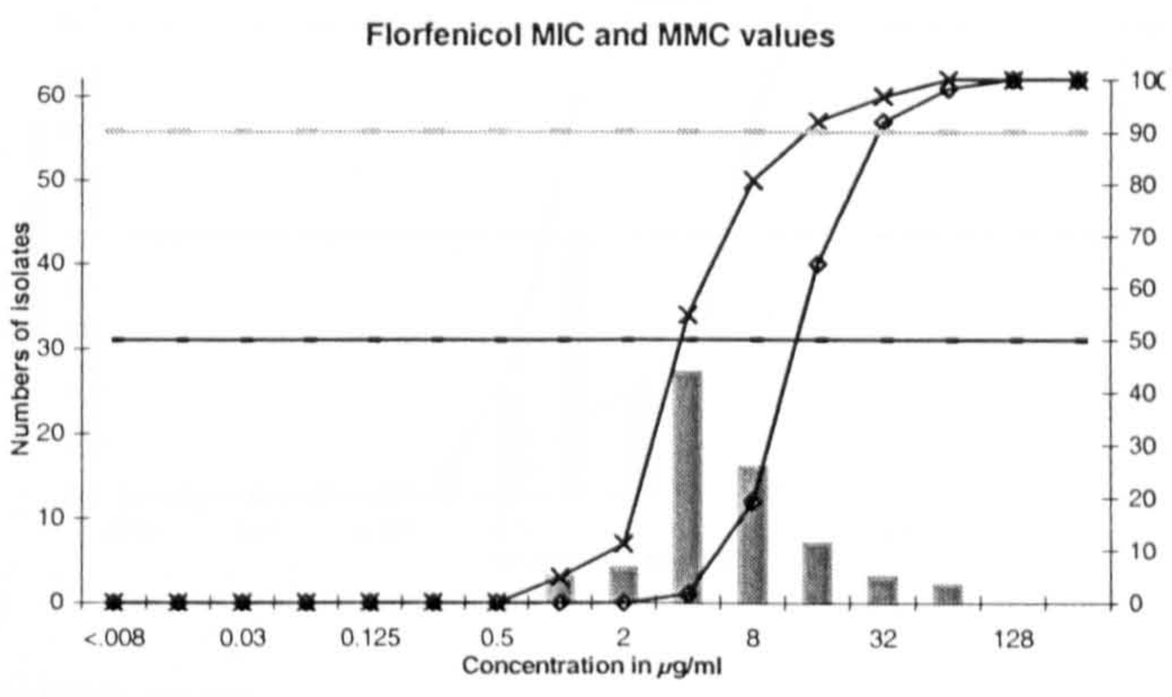
In conclusion the strategic use of antimicrobials may have a role in the control of CBPP, but it would need careful implementation and monitoring. A trial would need to be carried out in the field to evaluate the true effectiveness of use of antimicrobials as a means of control. CBPP is a major disease problem in Africa and currently only Northern Portugal is affected in Europe. Observation of the disease and the collection of more isolates are required to continue this study into improving control of CBPP.

FIGURE 3. 3. DISTRIBUTION OF MIC'S AND ACCUMULATIVE % MIC AND MMC VALUES FOR THE FIVE ANTIMICROBIALS TESTED AGAINST ISOLATES OF <u>M. BOVIS</u>









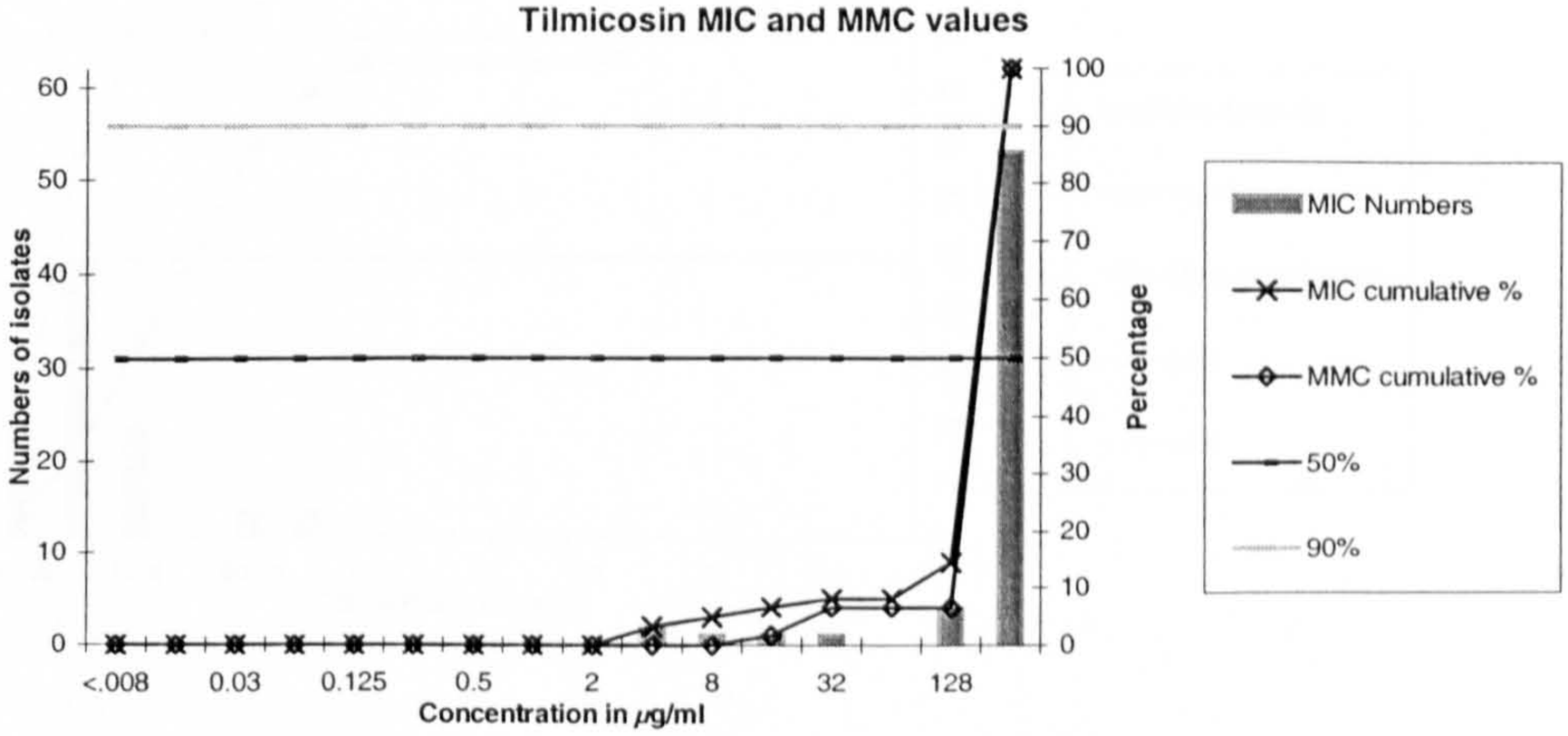
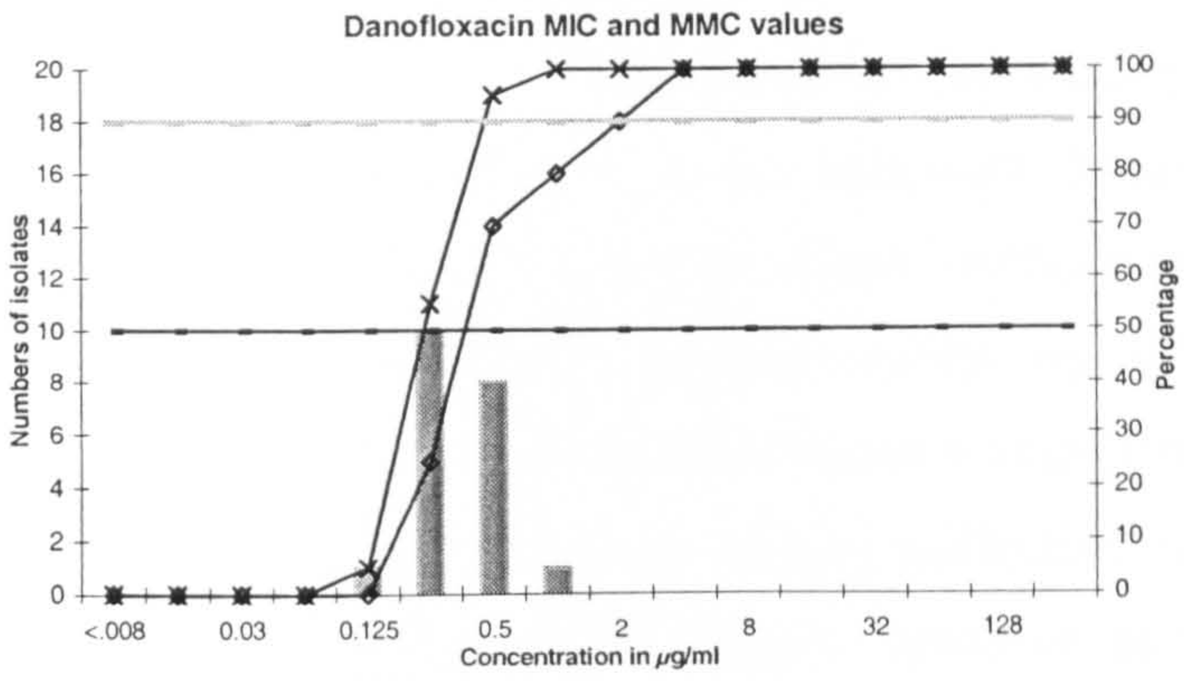
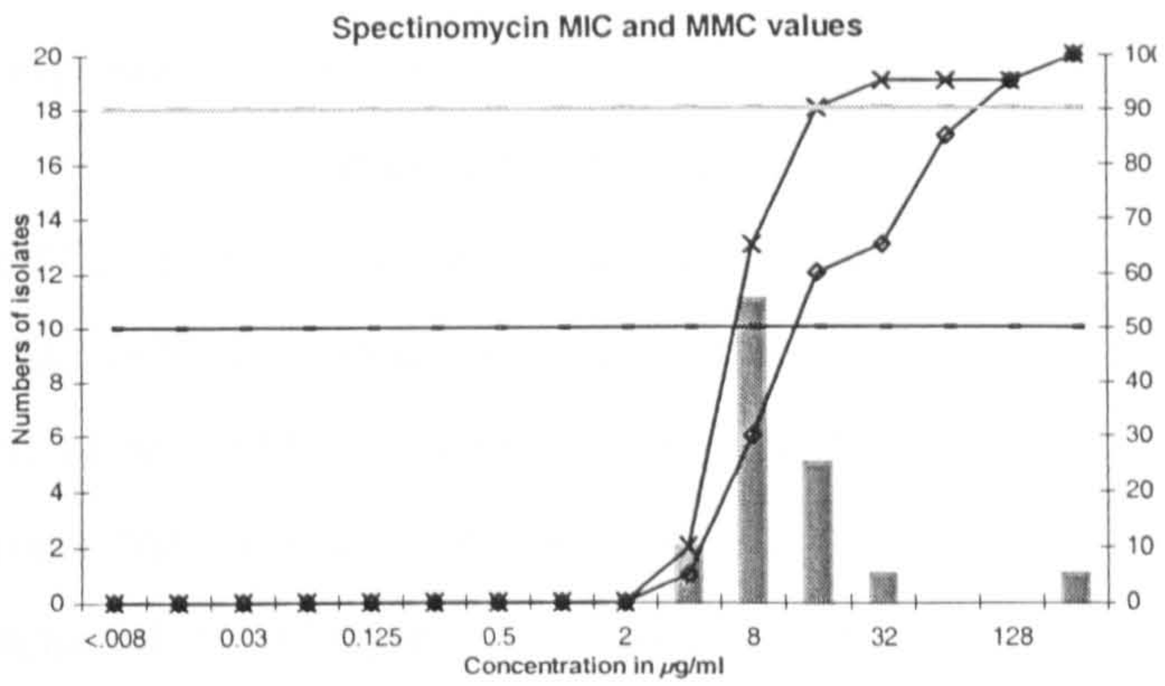
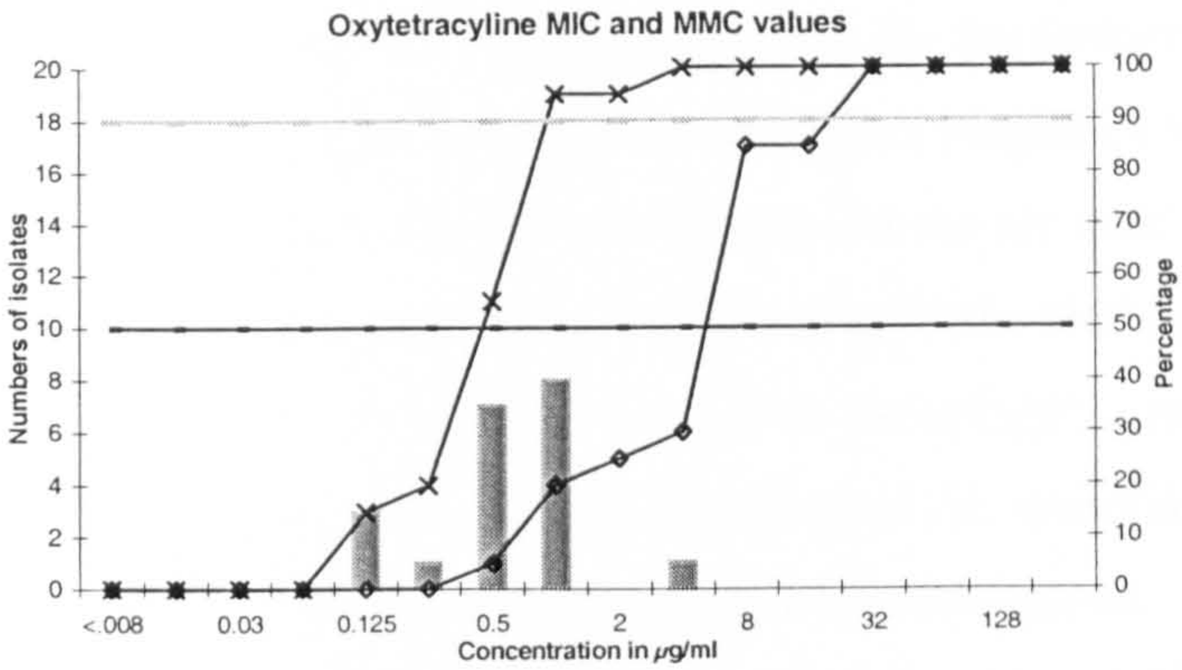
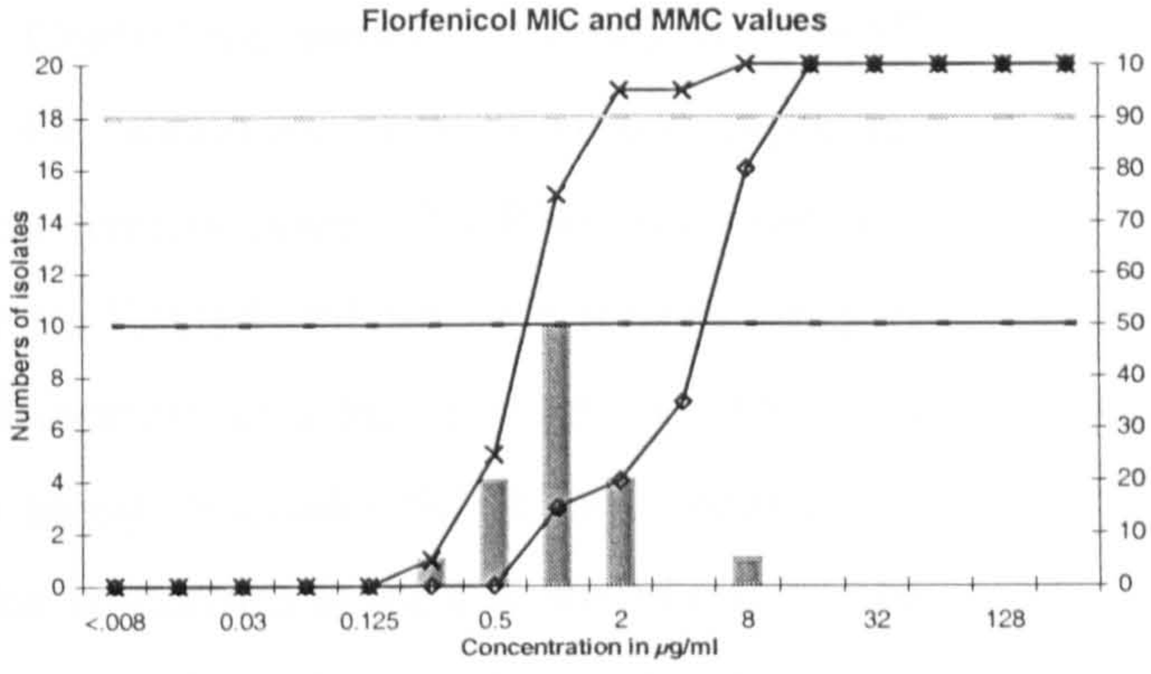


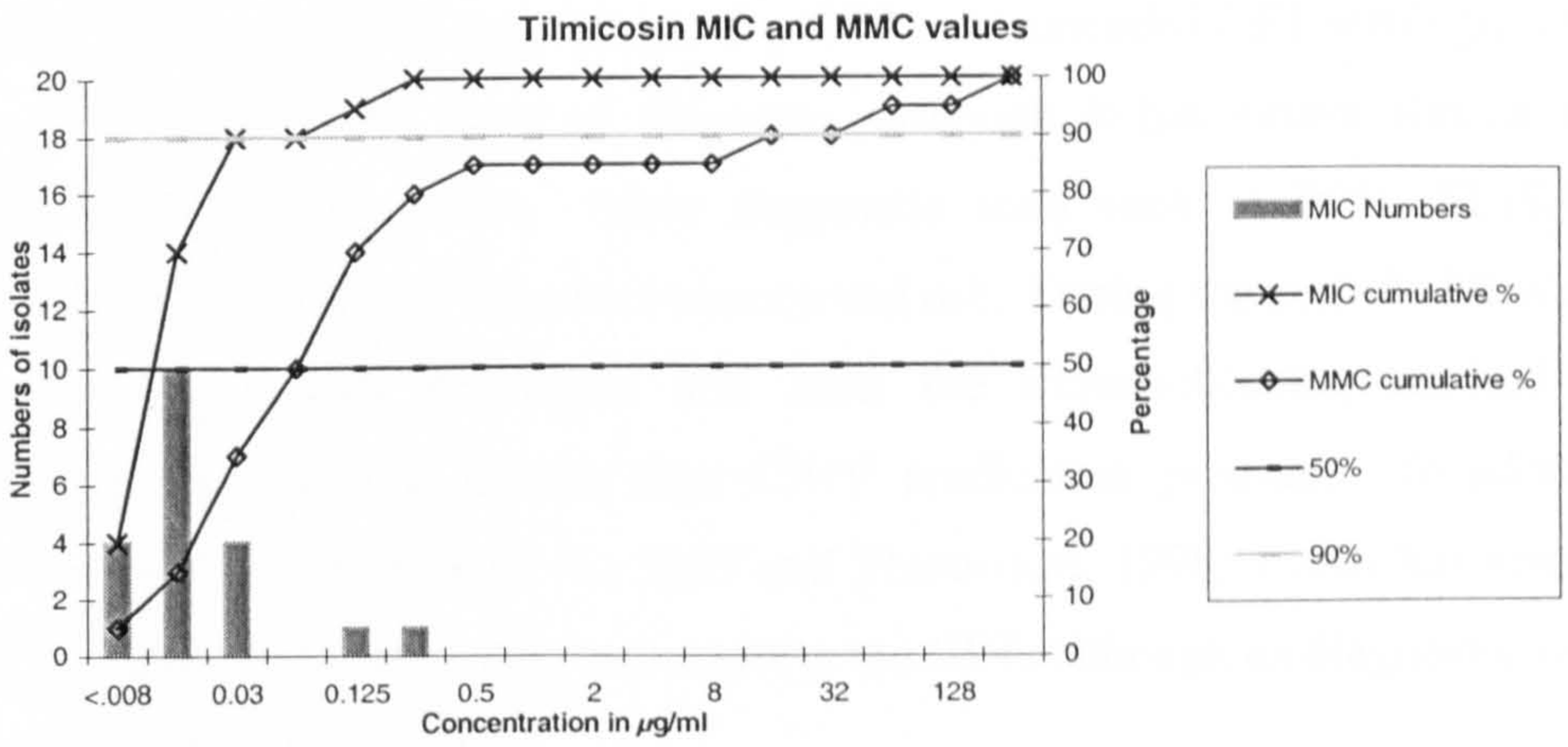
FIGURE 3. 4. DISTRIBUTION OF MIC'S AND ACCUMULATIVE % MIC AND MMC VALUES FOR THE FIVE ANTIMICROBIALS TESTED AGAINST ISOLATES OF M. MYCOIDES SUBSP. *MYCOIDES SC*











CHAPTER 4

4. 1. A CRITICAL EVALUATION OF DIAGNOSTIC METHODS FOR CBPP

4. 1. 1. INTRODUCTION

Contagious bovine pleuropneumonia, caused by *M. mycoides* subsp. *mycoides* SC, is an important disease. In affected countries, it causes great economic losses, as control methods are difficult to implement. Vaccination using attenuated *M. mycoides* subsp. *mycoides* SC cells is expensive and provides only transient protection of up to 1 year (Tulasne *et al.*, 1996). Antimicrobials have been considered to be largely ineffective or mask the disease symptoms so aiding the spread of the disease, however as discussed in Chapter 3, strategic use of antimicrobials may have a role in the future control of CBPP. Countries that have successfully eradicated the disease have all used a strict testing and slaughtering regime with compensation for the farmers. Control methods in countries with CBPP depend on correct and rapid diagnosis in the laboratory. It is therefore important that efficient and effective tests are used to correctly detect CBPP and the causative mycoplasma. Current diagnostic methods are limited and are described in Chapter 1, section 1.5.7. Gross pathology at post-mortem is a major diagnostic tool, but culture and identification of *M. mycoides* subsp. *mycoides* SC is fundamental, but seldom carried out in African countries. The authorities in the UK would require the causative organism *M. mycoides* subsp. *mycoides* SC to be isolated before implementing a slaughtering regime. The OIE recommended CFT serological test is currently the accepted form of diagnosis, although it has severe limitations as described in Section 1.5.7. Other diagnostic tests such as PCR, ELISA and immunoblotting methods are not in widespread use. During the period of this thesis, Portugal has further developed and used the immunoblotting method as a confirmatory diagnostic test in their CBPP eradication program. In addition a cELISA has been developed (Le Goff and Thiaucourt, 1998) which has now been accepted by the OIE as an alternative test to the CFT, although its diagnostic value is only comparable to the CFT.

In this study, samples were extracted from CBPP positive animals at a Portuguese abattoir when they were slaughtered. This material was used in this

study which critically evaluates the current CBPP diagnostic methods, in some cases with developments of methodology. Methods used were:

culture using established media

serological test by CFT using OIE standard antigen

gross pathology

immunocytohistochemistry (ICC)

PCR with Southern blotting detection.

In addition, observations were made on the farms involved in that specific CBPP outbreak which occurred in northwestern Portugal around Porto during January 1996.

4. 2. SOURCES OF CLINICAL MATERIAL

4. 2. 1. POST-MORTEM EXAMINATION AND SAMPLE COLLECTION

Twenty cows were examined for gross pathology in the abattoir in Porto. Selections of different samples were taken from different animals. Samples of lung were taken from eleven cows originating from a farm designated: '3' and immediately fixed in buffered formalin for ICC examination. Seven lung samples were taken from cows originating from three other farms (see Table 4. 1.) and were stored at -80°C before being tested by culture and polymerase chain reaction (PCR). Nasal swabs were taken from 16 cows and were immersed in Eaton's culture medium before being cultured and subjected to PCR.

4. 2. 2. OTHER SERUM SAMPLES

In addition to the sera collected from the abattoir, sera samples were also examined from six other Portuguese farms where CBPP had been diagnosed. Details of these samples are given in Section 6. 2. 1. 3.

4. 2. 3. MONOCLONAL ANTIBODIES

The Monoclonal Antibody Production unit at VLA (Weybridge) produced monoclonals against whole cells of *M. mycoides* subsp. *mycoides* SC Afadé strain for use in this work. Hybridomas were initially screened using a whole

cell antigen in an indirect ELISA. Following positive ELISA reactions, 19 hybridomas were selected for further evaluation.

The isotype for each Mab was determined using the Isodetect isotyping kit (Stratagene Ltd, Cambridge, UK) and following the kit protocol. Briefly the Mab supernatant was diluted 1:1 in PBS and 500 µl of sample slowly added to the sample well. Results were read within 5 minutes with a colloidal gold band indicating the specific isotype.

As an initial screening process, an *M. bovis* ELISA was performed, followed by western blotting (Towbin *et al,* 1979) using two mycoplasma isolates: *M. mycoides* subsp. *mycoides* SC 'Afadé'; and *M. bovis* '107B97' to check for cross-reacting Mabs and the size of the cross-reacting antigen. A 12 % SDS PAGE was run for the two isolates at a protein concentration of 500 µg/ml, before transferring to a membrane using a semi-dry blotting system ('Transblot', Bio-Rad Laboratories, Hemel Hempstead, UK). Control samples containing molecular weight markers and a strip of both antigens were stained with the protein dye, amido black (Sigma Chemicals, Poole, UK.). The remaining membrane was cut into strips and blocked with PBS pH 7.2 containing 3 % w/v dried milk powder (Marvel) and 0.05 % v/v tween 80 for 30 minutes at 37°C. This was followed by: incubation with the Mab supernatants for 60 minutes at 37°C; washing in PBS tween; incubation with anti-mouse polyvalent immunoglobulin labelled with alkaline phosphatase conjugate for 60 minutes at 37°C (Sigma Chemicals, Poole, UK.); washing four times in PBS; and addition of substrate 5 bromo-4-chloro-3-indoyl-phosphate/nitroblue tetrazolium (BCIP/NBT). The reactions were stopped by washing the strips in water after sufficient colour had developed in the control samples.

4. 2. 4. IMMUNOSTAINING OF LUNG TISSUE

Selection of Mabs for immunocytohistochemistry was carried out on formalin fixed bovine lung tissue. Those that failed to detect antigen or gave unwanted staining in normal lung tissue were considered unsuitable. Further evaluation of ICC using potentially suitable Mabs was carried out using tissue from 16 CBPP affected Italian cattle (Nicholas *et al.*, 1996), from normal animals and from archived cases of non-CBPP respiratory disease used as controls. The

histopathology unit at VLA (Weybridge) carried out this work. Mab M92/20 was identified as detecting specific antigen in lung tissue sections from CBPP affected animals using immunocytochemical staining; no staining was seen with the Mab in tissues from normal animals or those suffering other respiratory diseases.

Immunocytohistochemistry was undertaken basically following the method of Rodriguez *et al.* (1996c). Briefly, formalin fixed lung tissue from 11 Portuguese cows from this study were embedded in paraffin wax. Sections of 5 μm were dewaxed, rehydrated and endogenous peroxidase blocked. After appropriate pre-treatment, sections were incubated with normal calf serum to reduce non-specific reactions, and then incubated with the Mab M92/20. Signal amplification was performed using a sensitive avidin-biotin-peroxidase-complex (ABC) technique and immunodetection was visualised using diaminobenzidene. Sections were counterstained with Mayer's hematoxylin, dehydrated, cleared and mounted in DPX before examination by light microscopy.

4. 2. 5. CULTURE

Culture was carried out in Eaton's broth medium (section 2. 2. 2.) containing phenol red and the following antimicrobials: 'penbritin' (ampicillin) (Centaur Services, UK), 0.5 mg/ml; polymixin (Sigma, UK) 500 iu/ml; thallium acetate (Sigma, UK) 0.5 mg/ml; sulphadimidine (Sigma, UK) 0.5 mg/ml. Swabs or lung tissue were immersed in Eaton's medium and three 10 fold dilutions were made before incubation at 37°C in an atmosphere of 5 % carbon dioxide in air (carbon dioxide incubator; Hereus). Isolates were identified using conventional methods (see Section 1. 5. 7. 4.) and confirmed by PCR (Bashiruddin *et al.*, 1994b).

4. 2. 6. POLYMERASE CHAIN REACTION FOR <u>M. MYCOIDES</u> SUBSP. <u>MYCOIDES</u> SC

PCR was used to identify *M. mycoides* subsp. *mycoides* SC isolates (see Section 4.2.5.) and to detect *M. mycoides* subsp. *mycoides* SC specific DNA in nasal swabs and lung tissue. Nasal swabs were taken by inserting a sterile swab wetted in Eaton's medium approx. 15 cm into the nasal cavity. The swab was then removed and swirled vigourously in a bijou containing 3 ml of Eaton's medium.

DNA was extracted as described by Bashiruddin (1998). Briefly, cells were washed twice in TNE Buffer (0.01M Tris-HCL, pH8.0, 0.01M NaCl, 0.01M EDTA). They were resuspended in 200 µl of TNE buffer containing 10 % sodium dodecyl sulphate (Sigma, UK) and 10 % sarcosine (Sigma, UK); 20 µl of proteinase K 20 mg/ml in water (Sigma, UK) was added before incubation for 1 hour at 37°C. An equal volume of buffer saturated phenol (Gibco Life Sciences, UK) was added before centrifugation in a microcentaur centrifuge (MSE, UK) at 13,000 rpm for 5 minutes. The aqueous phase (supernatant) was carefully removed into another microcentrifuge tube before addition of an equal volume of phenol:chloroform:isoamyl alcohol (Gibco Life Sciences, UK) and further centrifugation as before. The aqueous phase (supernatant) approx 200 µl was again carefully removed into another microcentrifuge tube before precipitating the DNA by addition of 20 µl of 3.0M sodium acetate pH 5.5 (Sigma, UK) and 440 µl of cold 100 % ethanol (Sigma, UK). This was then frozen at -20°C overnight. The tubes containing the DNA were then centrifuged at 13,000 rpm for 5 minutes. The supernatant discarded and the DNA was washed in 70 % ethanol (Sigma, UK), followed by further centrifugation at 13,000 rpm for 5 minutes. The supernatant was carefully discarded and the DNA dried for one hour at room temperature before the addition of 100 µl of HPLC grade water to dissolve the DNA. This was then used as the DNA template in PCR.

Lung tissue was frozen at –80°C within three hours of removal from the carcases until it was examined in the UK. Five sections of lung tissue were 'chipped' off the frozen stored lung tissue to give small approximately 0.5 cm^3 which were placed in a mortar with 1ml of TNE buffer before being macerated with the pestle. Two hundred µl of this suspension was washed twice in TNE buffer before DNA was extracted following the method described above for nasal swabs.

The PCR used was based on oligonucleotides designed for the CAP 21 gene fragment (Taylor *et al.*, 1992) and specifically amplified a region of the gene present in strains of *M. mycoides* subsp. *mycoides* SC and LC and *M. mycoides* subsp. *capri*. The 534 bp amplicon was then cut using the restriction enzyme *Asn*I to specifically identify SC isolates (Bashiruddin *et al.*, 1994b) See figure 4.1.

FIGURE 4. 1. M. "MYCOIDES CLUSTER" PCR AND RESTRICTION ENZYME ANALYSIS WITH ASN1

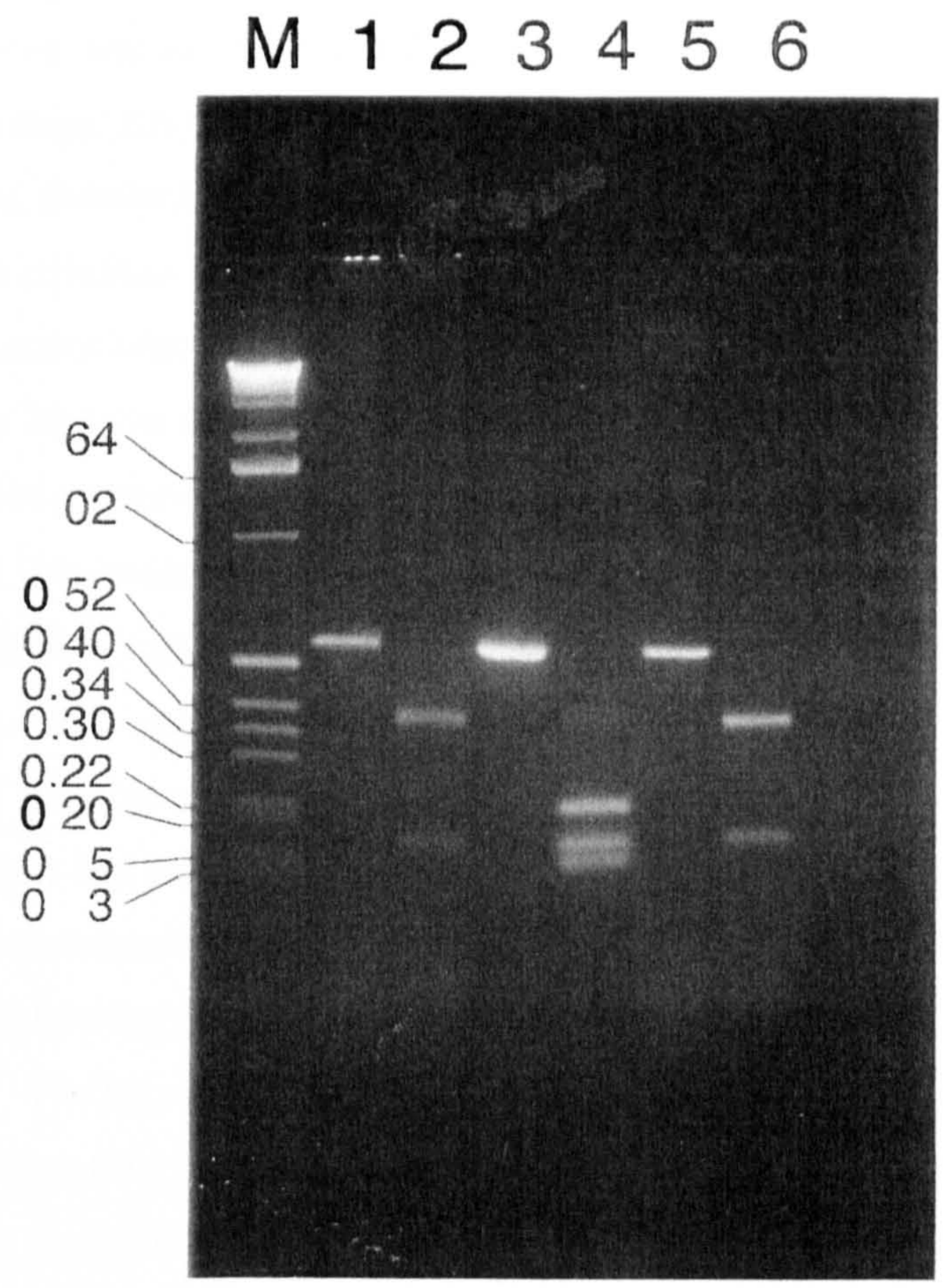


Figure shows M, molecular weight marker, 1, 3, and 5 uncut PCR products. Lanes 2 and 6 show *M. mycoides* subsp. *mycoides* SC following digestion with restriction enzyme *Asn*1. Lane 4 shows *M. mycoides* subsp. *mycoides* LC following digestion with restriction enzyme *Asn*1.

A Southern Blot method was used to determine if the sensitivity of the PCR amplicon detection could be increased. The Southern Blot was carried out using a P_{32} radiolabelled PCR product as a probe as described. The amplicon from the positive PCR control was run on a 3 % NuSieve agarose (Flowgen, UK) gel. The amplicon was excised from the gel, with the agarose blocks being placed in a microcentrifuge tube. The DNA was extracted using a sephaglass kit method (Pharmacia, Sweden). The DNA was quantified by mixing 5 μl of DNA with 5 μl of 2 μg/ml ethidium bromide and comparing the uv fluorescence from the spot with standard known concentrations (Maloy *et al.*, 1996). The DNA concentration was adjusted to 30 μg in 48 μl. The standard agarose gel that was run following the PCR from the lung tissue samples was placed in denaturing solution (8.77 % sodium chloride, 2.0 % sodium hydroxide) for 1 hour. This was replaced with neutralising solution (8.77 % sodium chloride, 6.65 % Trizma base, 0.2 % EDTA 0.5 M pH 8.0) for 1 hour. The gel was then placed on a plastic film (saren wrap). A piece of hybond N membrane cut to the size of the gel was placed on top followed by blotting paper and absorbent material. A heavy lead weight was added and left for 2 days. The membrane was then removed and fixed in uv light for 4 minutes. Unless stated the following prehybridisation and probing steps were all carried out at 65°C.

The membrane was rolled with a nylon support membrane and placed in a hybridisation tube (Hybaid, UK) and incubated for 30 minutes with 10 ml of rapid-hyb buffer (Amersham life sciences, UK). The DNA for labelling was heated at 95°C for 5 minutes and then placed on ice for 2 minutes. This was added to a tube of rediprime DNA labelling system (Amersham life sciences, UK) before addition of 2 μl of P_{32} radiolabel. This was centrifuged and then incubated at 37°C for 10 minutes followed by 95°C for 1 minute. This was centrifuged briefly before using a ProbeQuant G-50 Micro Column (Pharmacia, Sweden), which was briefly centrifuged again. This retained the unattached radiolabel in the column, leaving the labelled DNA in the tube, which was then placed in the hybridisation tube with the membrane and incubated for 2 hours.

The membrane was then washed for 20 minutes with 100 ml of 2 x SSC (1.75% sodium chloride, 0.88% sodium citrate, adjusted to pH 7.0) with 1% SDS, followed by two further washes for 15 minutes. The membrane was then removed

and placed on blotting paper to dry before wrapping in saren wrap and being placed in a film cassette with film. The film was left for 2 hours at –70°C before being developed.

4. 2. 7. SEROLOGY

The CFT was carried out in the serology section at VLA (Weybridge) using the standard method of the OIE, (1996) with antigen obtained from LNIV (Portugal).

An indirect ELISA using a whole cell antigen of *M. mycoides* subsp. *mycoides* SC strain B103 was used to test the sera obtained. The ELISA protocol was similar to that described in Section 5.2.7. The antigen was prepared by washing cells three times in PBS and the microtitre plates were coated with antigen at a concentration of 1.5 μg/ml in bicarbonate/carbonate buffer. Sera were tested at a dilution of 1 in 320.

4. 3. RESULTS

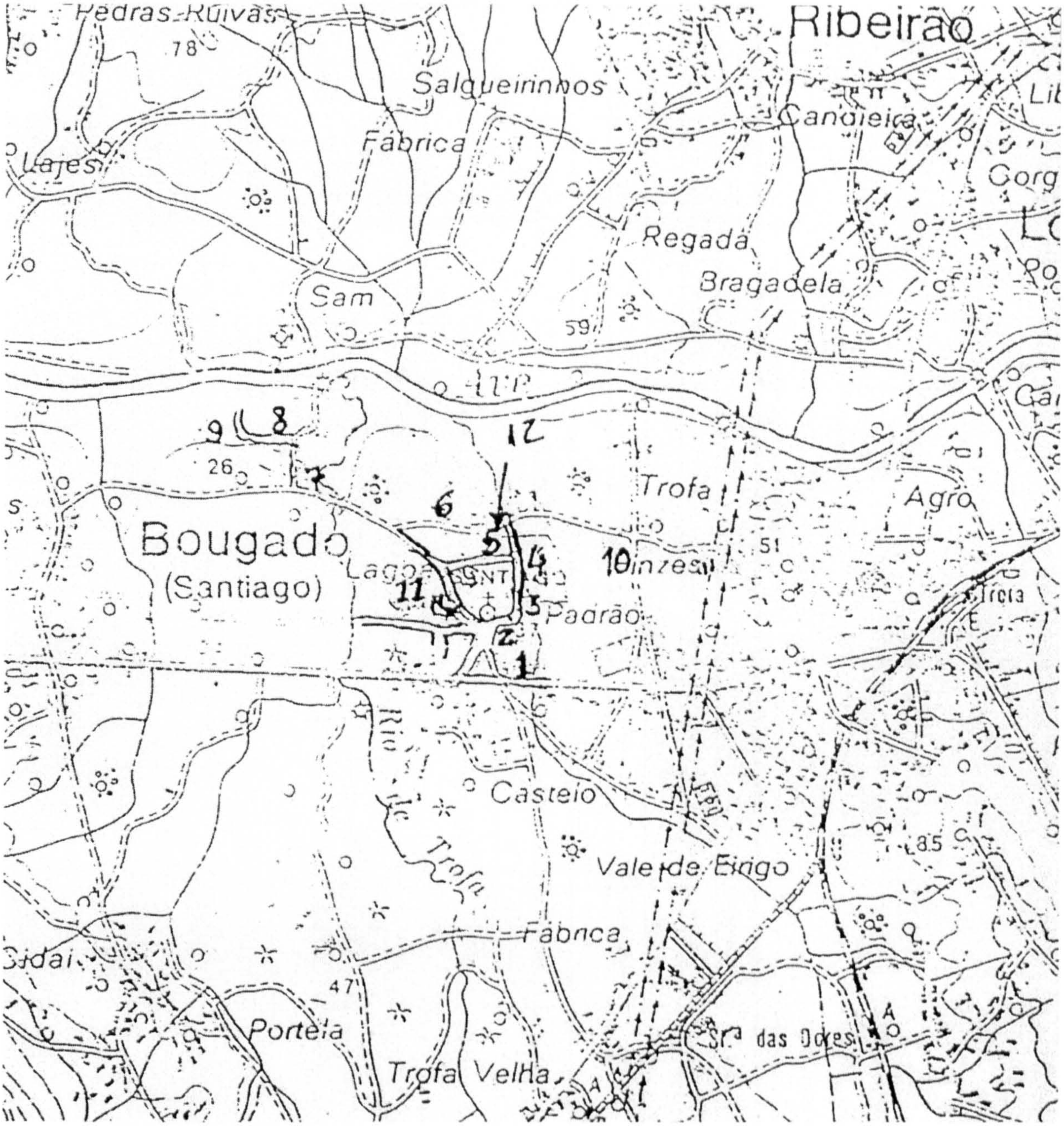
4. 3. 1. OBSERVATION ON CATTLE FROM CBPP INFECTED HERDS PRIOR TO SLAUGHTER

Two CBPP affected herds from a single village approximately 30 kilometres north east of Porto (Figure 4. 2), were studied. The first outbreak in the village was on 'farm 3'. Subsequently the 12 other herds within the village were closely monitored, but only one other herd, at 'farm 4' became affected. CBPP in the first herd was diagnosed by the CFT and confirmed by immunoblotting (Regalla *et al.*, 1996b). Milk production had fallen and the cows were not 'thriving', showing slight loss of condition with a dull coat and heads slightly lowered. They showed mild clinical signs with occasional coughing when moved. One case of abortion was reported on the farm. The cows were kept in a semi-open barn with a fenced concrete yard. On one side of the barn and yard was a breeze block wall which was shared with the neighbouring farm. The neighbouring farm, farm '4', kept cows in a semi-open barn with the shared breeze block wall forming the back of the barn. The barn was covered with a loose fitting corrugated roof that had large gaps where it joined the shared wall. The prevailing wind during the period of investigation meant

that farm '4' was upwind of farm '3'. This farm had purchased cattle from a dealer at farm '5' which was situated immediately opposite the entrance to farm '4'. CBPP was not detected on farm '5'.

All the results are summarised in Table 4. 1. All diagnostic tests were conducted at VLA (Weybridge).

FIGURE 4. 2. LOCATION OF CBPP OUTBREAKS IN BOUGADO, PORTUGAL



The farms monitored in Bougado were numbered 1-12. Farm '3' was the first farm where CBPP was detected; possible sources were farm '4', and farm '5'. The arrow shows the prevailing wind direction.

TABLE 4. 1. EVALUATION OF DIAGNOSTIC TESTS ON CATTLE SLAUGHTERED IN PORTUGAL DURING JANUARY 1997

Abattoir No.	Animal No.	Farm	Lesions	CFT	ELISA	ICC	Nasal Swabs		Lung (frozen)	
							Culture Eaton's	PCR (direct)	Culture	PCR
016	VLA 1	NK	Positive	NT	NT	NT	Negative	Negative	Negative	Negative
397	B315426	A	Positive	1:40	0.72	NT	Negative	Positive	Negative	Positive*
398	VLA 2	NK	Positive	1:20	0.77	NT	Negative	Positive	Negative	Negative
400	B554513	B	Positive	NT	NT	NT	Positive	Positive	Positive	Positive
403	B311198	C	Positive	1:160	0.82	NT	Positive	Negative	Positive	NT
406	B311403	C	Positive	NT	NT	NT	NT	NT	Negative	Positive
410	VLA 3	NK	Positive	1:80	0.60	NT	NT	NT	Positive	Positive
412	B077852	C	Positive	1:80	0.92	Positive	Positive	Positive	NT	NT
413	B077820	C	Positive	1:10	0.65	Positive	Negative	Positive	NT	NT
414	B075328	C	Positive	1:20	0.67	Positive	Negative	Negative	NT	NT
415	B077956	C	Positive	NT	NT	Positive	Positive	Negative	NT	NT
416	B077802	C	Positive	1:40	0.76	Positive	Negative	Negative	NT	NT
420	B077840	C	Positive	1:40	0.84	Positive	Negative	Positive	NT	NT
421	B185267	C	Positive	1:20	0.60	Positive	Negative	Positive	NT	NT
422	B075318	C	Positive	1:40	0.70	Positive	Negative	Positive	NT	NT
424	B311096	C	Positive	1:640	NT	Positive	Positive	Negative	NT	NT
425	B185268	C	Positive	NT	NT	Positive	Negative	Negative	NT	NT
426	B077843	C	Positive	1:80	0.66	NT	NT	NT	NT	NT
427	B185331	C	Positive	1:40	0.95	Positive	Positive	Negative	NT	NT
434	B376689	D	Positive	NT	NT	NT	NT	NT	NT	NT

A. Victor Manuel Andrade Martins (Farm not in Bougado)

B. José Luís Sá Torres (Farm 4 in Bougado)

C. Soc. Agric. Grupo Lagoa (Farm 3 in Bougado)

D. Gabriel Araújo Martins (Farm not in Bougado)

* Positive only using DNA probe

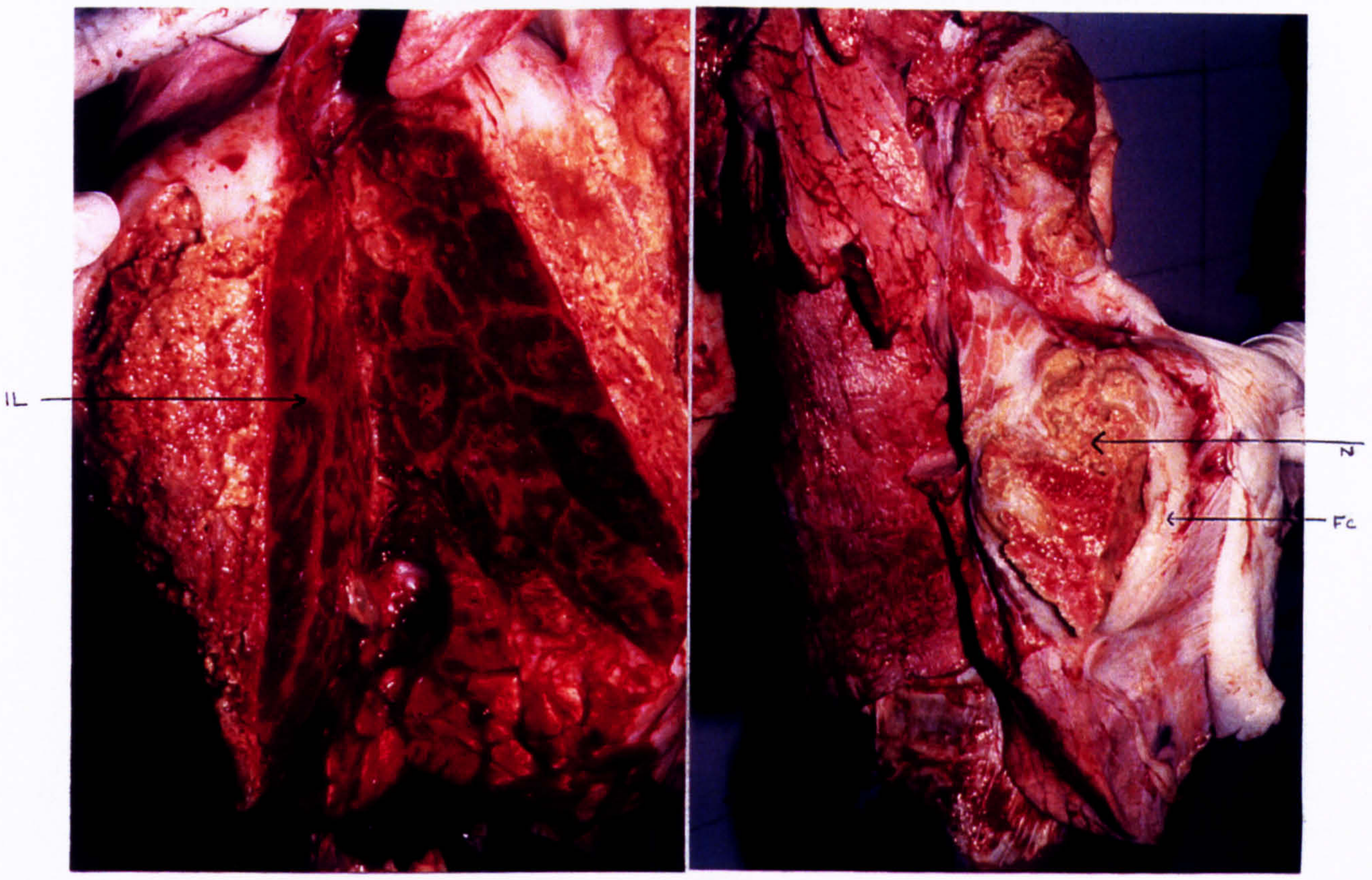
NT: NOT TESTED

NK: NOT KNOWN

4. 3. 2. GROSS PATHOLOGY OF SLAUGHTERED ANIMALS

Twenty cows were examined. All showed gross lung lesions varying in size from 10 cm to a whole lung. Nineteen had lesions or sequestra present on only one of the lungs (unilateral) whilst one cow had a small sequestra on the second lung. The affected lungs showed chronic signs of infection with necrotic tissue encapsulated by fibrous connective tissue or sequestra. In two of the cows, the infected lungs were almost completely necrotic. Adhesions of the lung to the body wall were also present in some of the carcasses.

FIGURES 4. 3. CBPP GROSS PATHOLOGY OBSERVED AT POST-MORTEM



Acute CBPP lesion after incision. Hepatisation of lobules is evident with oedema and thickening of interlobular septa (IL) giving 'marbled' appearance.

Chronic CBPP lesion: incision of sequestra showing fibrotic capsule (FC) containing necrotic lung tissue (N).

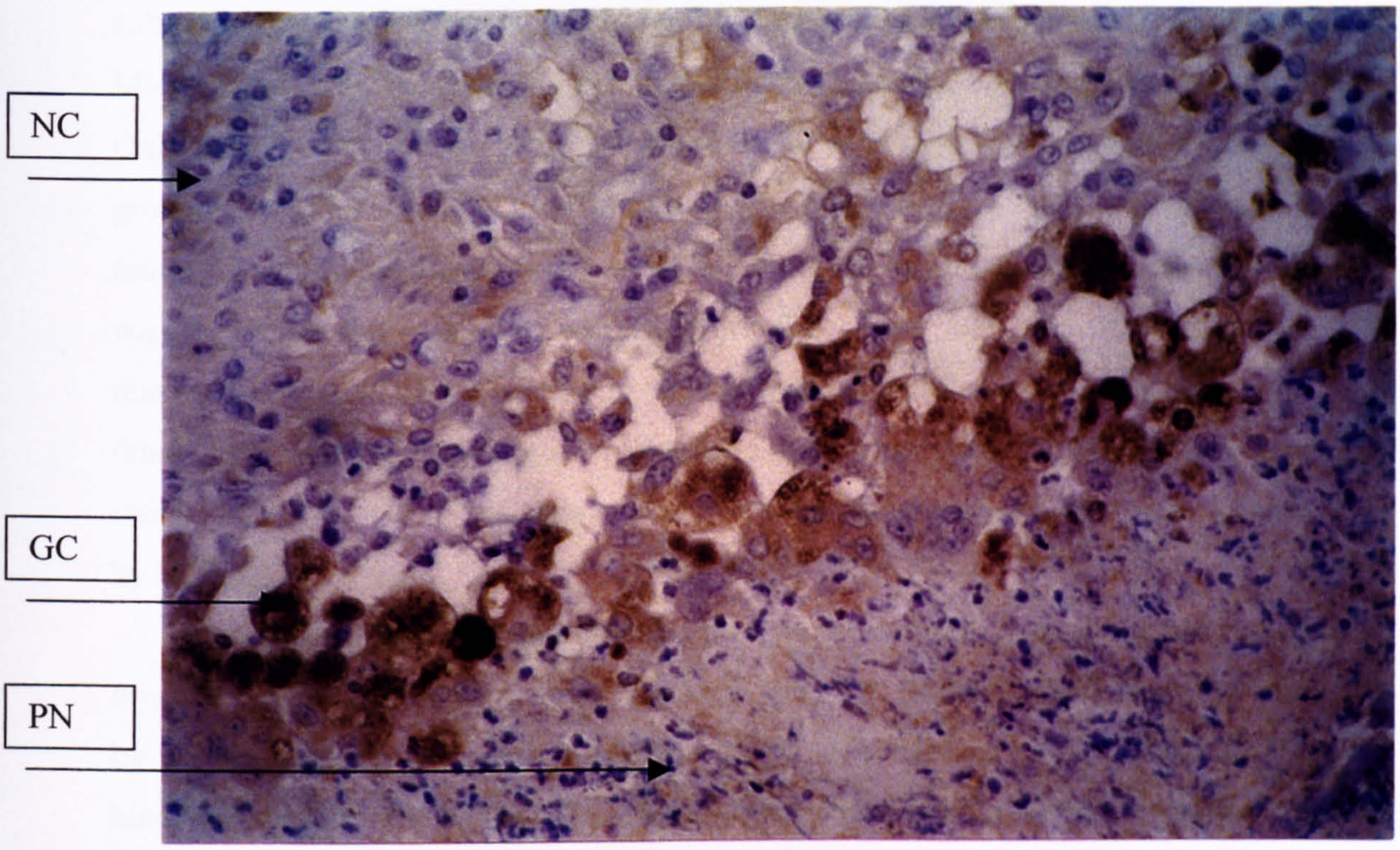
4. 3. 3. MONOCLONAL ANTIBODIES

The initial screening using the *M. bovis* ELISA and western blotting with the *M. bovis* antigen showed reactions with nine of the 19 Mabs, making them unsuitable for use. Some Mabs proved unsuitable for use for immunohistochemistry, due to failure to detect antigen, or presence of unwanted staining in normal lung tissue. The Mab 92/20 was selected for use in immunocytohistochemistry because it appeared specific in tests and gave clear backgrounds in normal tissue. This Mab, 92/20 is IgG1 sub class k, and gave an OD_{450} of 0.66 in the *M. mycoides* subsp. *mycoides* SC ELISA, OD_{450} 0.08 in the *M. bovis* ELISA, but was very unusual in giving many diffuse bands with western blotting. It was shown to react in the latex agglutination test (see chapters 5 and 6) suggesting it may be raised against the capsular polysaccharide of *M. mycoides* subsp. *mycoides* SC.

4. 3. 4. IMMUNOCYTOCHIEMISTRY

All eleven samples of lung tissue examined by this method were positive. Using the specific Mab 92/20, antigen was clearly visible in tissue sections with minimal background staining (Figure 4. 4).

FIGURE 4. 4. SECTION OF LUNG FROM CBPP INFECTED ANIMAL STAINED USING Mab 92/20



Section of lung from CBPP affected cow. Stained section shows lesion with: pyknotic nuclei (PN) (right); giant (epithelial) cells (GC) in middle; normal cells (NC) (top left). Areas where antibody bound to cells are stained brown. Nuclei are stained blue/purple.

4. 3. 5. CULTURE AND PCR

The three 10-fold dilutions from each sample were examined daily for up to 2 weeks, unless a mycoplasma species was isolated earlier, in which case further examination of cultures from that cow was discontinued. Cultures showing bacterial contamination were filtered through a 0.45 μm filter so that approximately 100 μl was used to seed a fresh Eaton's broth. Otherwise any observed growth was sub culturing into a fresh broth before being identified (see section 2.1.2.). Microbial growth was observed in cultures from 15 of the 16 nasal swabs examined. Only six of these isolates were identified as *M. mycoides* subsp. *mycoides* SC by growth inhibition test (GIT) and these were confirmed using PCR. *Acholeplasma laidlawii* was isolated from one sample where no *M. mycoides* subsp. *mycoides* SC was recovered and was identified using specific rabbit antiserum in a GIT. The remaining eight cultures were of other cell-walled bacterial species. PCR carried out directly on nasal swabs detected *M. mycoides* subsp. *mycoides* SC in eight of the 16 swabs; however, four of the six samples that were positive by culture were negative by PCR.

Seven frozen lung tissue samples were cultured for *M. mycoides* subsp. *mycoides* SC and six of these were also tested by PCR. Three samples were positive by culture, and all were then confirmed as *M. mycoides* subsp. *mycoides* SC by biochemical tests and GIT. The two culture positive samples tested were also positive by PCR. However, the direct PCR on lung tissue was also positive for three samples including one that was culture negative. Use of Southern blotting further increased the PCR detection level to include another positive, giving four out of the six samples as positive.

4. 3. 6. SEROLOGY

Blood was available from thirteen of the slaughtered animals. These all gave positive results by both ELISA and CFT. The ELISA results were all clear positives with OD_{450} values ranging from 0.60 to 0.95 with the negative control giving a value of less than 0.15. The CFT gave a range of titres from 1:10 to 1:160. However, there was little correlation between the two tests indicating that the tests were measuring different classes of antibody.

Although the CFT is the standard test, there is substantial evidence that it fails to detect infected animals in the early sub-clinical stages of the disease (Stärk *et al.*, 1994). Therefore a more comprehensive evaluation was undertaken using 169 samples from 6 farms where there was evidence of CBPP infection. The results are given in Table 4.2.

TABLE 4. 2. COMPARISON OF CFT AND IMMUNOBLOTTING TESTS FROM SIX CBPP AFFECTED FARMS IN PORTUGAL

FARM	NUMBER OF ANIMALS	NUMBER OF IMMUNOBLOT POSITIVES	NUMBER OF CFT POSITIVES
1	55	47	29
2	25	12	9
3	18	17	11
4	19	7	6
5	15	3	0
6	37	35	18

These results clearly demonstrated the increased sensitivity of immunoblotting compared to the CFT with 121 (71.6 %) positives by immunoblotting compared with 73 (43.2 %) by CFT.

4. 4. DISCUSSION

In CBPP infections the gross lesions and sequestra, usually present on a single lung, are pathognomonic (Nunes Petisca *et al.*, 1990), although inexperienced workers may mistake these for acute cases of pasteurellosis, actinobacillosis and *Echinococcus* which affects both lungs. Consequently isolation of the mycoplasma is necessary for confirmation. Recovery of the organism by culture is dependent on the type of sample taken, the culturing methods and the contaminating organisms present. In this study the effect of the time taken to travel from the abattoir in Portugal to the laboratory in England also adversely affected the success rate of

culture. Similar problems may also be expected in Africa where there are large distances between the herd and the diagnostic laboratory. In a study on CBPP in Italy (Nicholas *et al.*, 1996) only 10 isolates of *M. mycoides* subsp. *mycoides* SC were recovered fron 37 affected cattle. They stated that a loss of viability during transit, or overgrowth of the agent by contaminants including bacteria and acholeplasmas during culture, or suppression by antibacterial or inflammatory compounds may account for the low isolation rate.

Use of serological testing is beneficial for diagnosis. However serological testing is not always effective. The tests used are not conclusive as false results do occur, either with cross-reacting antibodies or failure to detect individual animals at certain stages of infection. Animals that have just been exposed to infection or those exposed previously may go undetected. In this study, (Table 4.2.) the CFT was insensitive when compared to the immunoblotting method. However, when used to detect a CBPP positive herd the CFT may be more suitable for use as a screening tool as immunoblotting is costly to perform and requires specialist interpretation. However in farm 5, Table 4.2. no cattle were positive by CFT on that farm, although 3 were positive by immunoblotting. This could have serious implications on a CBPP erradication scheme, as the disease would go unchecked and cattle movements would aid the spread of disease. In Portugal, farms are frequently small, many with just a few cattle. In these cases the number of samples tested may be too small for an insensitive serological test to detect CBPP positive animals. This may account for the continuing survival of CBPP in Portugal, although all cattle on a small farm can be tested in comparison with just a percentage of cattle on a larger farm. It is therefore essential to utilise different methods for confirming disease diagnosis.

PCR was used successfully for confirming the identification of isolates in this study. However, PCR was not totally effective in the detection of *M. mycoides* subsp. *mycoides* SC DNA, detecting only half of nasal swabs and lung tissue of infected animals. This failure of PCR to detect *M. mycoides* subsp. *mycoides* SC DNA may be related to the sensitivity of the PCR, the DNA extraction methods and PCR amplicon detection methods. The use of Southern blotting detection in this study increased the detection of amplicons and therefore the

sensitivity of the PCR. However, PCR was still not totally effective which may be due to the intermittent excretion of *M. mycoides* subsp. *mycoides* SC from the nasal passages, and the correct selection of a region of affected lung tissue where *M. mycoides* subsp. *mycoides* SC could be detected; other unknown inhibitory factors may affect the PCR. Verdin *et al.*, (2000) using a PCR detection system for *M. hyopneumoniae* reported that heme, heparin, polysaccharides and highly glycosylated molecules such as those found in mucus are known to inhibit PCRs.

Isolation and identification of *M. mycoides* subsp. *mycoides* SC provides definitive evidence that animals are infected, as serological tests may give false positive results and gross pathology may be ambiguous. In the event of an outbreak in the UK the control authorities would only slaughter if the mycoplasma was isolated. Consequently the need to improve cultural techniques is paramount. This study demonstrates the inadequacy of cultural and even PCR methods as only 39 % of nasal swabs were positive on culture, rising to 50 % using PCR. This also demonstrates the increased sensitivity and potential use of PCR in comparison with culture. However, results of culture and PCR detection may be improved by identifying regions of affected lung that may have increased concentrations of the target organism. In addition, developments are needed in sample handling and in the design of transport and culture medium that may include selective inhibitors to improve isolation and detection rates.

Histology and, to an increasing extent, ICC is used to confirm the diagnosis of CBPP particularly where the causative organism, *M. mycoides* subsp. *mycoides* SC, is not recoverable, where the animal has died of an acute disease, or when serology is not possible or unclear (Ferronha *et al.*, 1988; and Scanziani *et al.*, 1991). However the sensitivity of ICC using polyclonal serum can be low and non-specific results frequently occur (Nicholas and Bashiruddin, 1995). However, Di Francesco *et al.* (1996) showed good agreement between CFT, culture and pathology on 11 CBPP positive samples from Italy. The use of Mabs has helped to overcome these problems.

In this study, all 11 affected lungs examined by ICC using Mab M92/20 were positive for CBPP, whereas *M. mycoides* subsp. *mycoides* SC was isolated from primary culture in only four of those eleven cases and specific PCR amplicons

were detected in five. Thus, ICC successfully identified the presence of the mycoplasma in these cattle, confirming the gross pathology, CFT, immunoblotting and ELISA results. The main disadvantage with ICC is that the animal is already dead, although few tests are effective once an animal is dead.

Differences in virulence between African and European isolates of *M. mycoides* subsp. *mycoides* SC have been reported (Provost *et al.*, 1987) and morbidity and mortality are lower in European than in African disease outbreaks (Nicholas *et al.*, 1996). The difficulty in reproducing disease in experimental infections with European *M. mycoides* subsp. *mycoides* SC strains (Abdo *et al.*, 1998; Pini *et al.*, 1999) may also indicate a reduced virulence compared to African strains. However the pathological lesions caused during natural outbreaks in both Africa and Europe are identical and follow a similar pattern: during new outbreaks, many cattle show acute and sub acute lesions while chronic lesions and sequestra predominate in later outbreaks. The striking difference is the almost complete lack of mortality and obvious morbidity seen in European outbreaks which must be due in part to improved husbandry and better health of the cattle (Nicholas and Palmer, 1994).

In this study all serological tests were effective when used on the samples from the abbatoir, mainly because of the advanced progression of the disease in the herd. However, when used on the farm samples, serological tests were insensitive with the CFT detecting 48/169 (28 %) fewer positive samples than immunoblotting. A test such as the CFT, which is known to be only 70 % sensitive in the early and later stage of the disease, will fail to detect partially affected herds, as seen in Table 4.2. A very sensitive primary screening test, which unavoidably produces false positive results, backed up by a highly specific test such as immunoblotting is required to ensure detection of all affected herds. In Africa, a third test, which can be performed 'at the penside' is also required. Development and evaluation of such a test is described in Chapters 5 and 6.

CHAPTER 5

5. 1. DEVELOPMENT OF A LATEX AGGLUTINATION TEST FOR CBPP

5. 1. 1. INTRODUCTION

Microsphere or latex agglutination tests (LAT) have been used since 1956 when a rheumatoid factor test was developed (Singer and Plotz, 1956). In these tests polystyrene microspheres are used for the solid support of either antigen or antibody which are adsorbed onto them. These 'sensitised' microspheres act to amplify any antigen/antibody reaction that occurs, causing clumping of the microspheres in a positive test, which can be seen by eye. LAT's are portable, rapid, and efficient, and can be used in even the most primitive conditions, providing a diagnostic result within 2 minutes of starting the test.

Currently most LAT's use 0.2 – 1.0 μm diameter microspheres. It is thought that the larger the microsphere the more sensitive the test, based on assumptions about the number of clumps and therefore bonding required to be seen by eye. Theoretical sensitivity of a 1.0 μm diameter microspheres has been calculated to be as low as 10 pM (Bangs, 1996). In practice Tilton (1987) determined levels of bacterial polysaccharides detected by antibody coated microspheres were as low as 0.1 ng/ml.

Antigen or antibody can be used to coat the latex particles, which can be achieved by passive or physical methods. Passive methods utilise the hydrophobic properties of of the latex particles, which absorb proteins strongly and irreversibly, via the hydrophobic domains in the proteins, which may be affected by pH. However, covalent attachment may be required for small molecules with weak adsorption characteristics (Bangs, 1996).

The number of applications of the LAT has increased dramatically in recent years, mainly due to the development of specific Mabs and purified antigen extracts. These include more than 100 infectious diseases covering, bacterial, viral, fungal and protozoan infections, such as leprosy, cholera, brucellellosis, HIV, rinderpest, aspergillosis, and amoebiasis. Other LATs have been developed to detect chemical analytes, such as used in detection of drug abuse and antimicrobials in milk (Bangs, 1996).

Rurangirwa *et al.* (1987b) developed a LAT for field diagnosis of contagious caprine pneumonia caused by *M. capricolum* subsp. *capripneumoniae.* In this test mixed polysaccharides extracted from the mycoplasma culture supernatants were adsorbed onto 1.08 μm latex beads (Sigma Chemicals). The mixed polysaccharide consisted of glucose, galactose, mannose, fucose, glucosamine and galactosamine. One ml of latex beads was mixed with 1 mg polysaccharide in 1 ml phosphate buffered saline pH 7.4, containing 0.2 % sodium ethylene diamine tetra-acetate and 0.01 % sodium azide. This mixture was shaken and incubated at 37°C for 1 hour and 8ml of the same buffer added. LAT was carried out using 10 μl volumes of serum and latex beads.

M. capricolum subsp. *capripneumoniae* is a member of the *M. mycoides* cluster and is therefore closely related to *M. mycoides* subsp. *mycoides* SC. This suggests that it might be possible to use a *M. mycoides* subsp. *mycoides* SC polysaccharide extract in the development of a test to detect CBPP. However, there are conflicting reports as to the antigenic activity of polysaccharides from *M. mycoides* subsp. *mycoides* SC.

Kurotchkin (1937) first reported the isolation of a specific carbohydrate substance from *M. mycoides* subsp. *mycoides* SC. He also determined that large quantities of that carbohydrate were present in the culture medium and in the blood of animals severely affected by CBPP. Buttery and Plackett (1960) analysed carbohydrates from *M. mycoides* subsp. *mycoides* SC strains. They found carbohydrates accounted for approximately 10% of the dry cell weight and galactan was the main product obtained by extraction with warm aqueous phenol. When hydrolysed by acid it released galactose and approximately 4% of a soluble lipid. They thought the substances were probably haptens (small non-protein parts of certain antigen molecules that carry the chemical group that combine specifically with an antibody rather than complete antigens), as they observed no antibody responses after injecting galactan into experimental animals. Further analysis by Plackett and Buttery (1964) identified the structure to be consistant with 6-O-β-D-galactofuranosyl-D-galactose.

The ability of the polysaccharide to be used as an antigen in diagnostic tests has given conflicting results. The original carbohydrate extract from

Kurotchkin (1937) was active in precipitin tests but did not fix complement with bovine antisera. Dafaala (1957, 1959) separated two polysaccharide antigenic fractions, 'A' and 'B'. Fraction 'A' was found in cells, culture supernatant, exudates and blood of moribund animals and was similar to that obtained by Kurotchkin in that it did not fix complement. Fraction 'B' was found attached to cells only, and was active in both precipitin and complement fixation tests. White (1958) used immunodiffusion tests to demonstrate the presence of three soluble antigens in extracts of *M. mycoides* and in material from lesions and exudates. These three antigens may be similar to those obtained by Yoshida (1961) who fractionated *M. mycoides* cells into protein, carbohydrate and lipid. He found the lipid factions contained complement-fixing antigens, whereas the protein and carbohydrate did not. From this it appears that the pure polysaccharide extract cannot fix complement but displays some antigenicity in precipitin tests. Interestingly, Knight and Cowan (1961) prepared an antigen by the method of Dafaala and found it did not fix complement unless a heat labile factor present in normal bovine sera was added. It appears that the method of preparation, the purity of the polysaccharide extract, and the test used is an important factor in determining the usefulness of *M. mycoides* subsp. *mycoides* SC specific polysaccharides in diagnostic tests. March *et al.*, (1999) analysed the capsular polysaccharides of *M. mycoides* subsp. *mycoides* SC and concluded it comprised of mannose, glucose, galactose, fucose, glucosamine and galactosamine sugars. However, this contradicted previous findings by other workers.

There is an urgent need for a rapid field test for detecting CBPP, particularly for use in Africa where nomadic movements of cattle and large distances make laboratory testing impracticable. The current CFT (Campbell and Turner, 1953) is the recognised antibody detection test and is laborious and needs to be carried out in a laboratory. Slide agglutination tests have been developed for CBPP (Perreau, 1963; Priestly, 1951; Turner and Etheridge, 1963) and are used with either serum or whole blood. However, they are only useful on a herd basis as they give false positives at low serum dilution and are reliable only early in infection (Rurangirwa, 1995). Thus, work to develop a LAT was undertaken. The antigen used in development of the test was a carbohydrate extract, as that was successfully

used by Rurangirwa *et al.* (1987b) in the development of a field test for CCPP. However, it was recognised that there were conflicting reports as to the antigenicity of carbohydrate extracts from SC strains.

5. 2. MATERIALS AND METHODS

5. 2. 1. MEDIA

Except when stated Eaton's medium was used (see Section 2. 2. 2.). Carbohydrate production in this medium was also compared with that in PRM medium (Rice *et al.*, 2000). The composition of PRM medium is: special peptone L-72 (Oxoid) 20.0 g/l; yeast extract (Oxoid) 5 g/l; fresh yeast extract 100 cm^3/l; glucose 5.0 g/l; pyruvate 2.0 g/l; glycerol 5.0 g/l; sodium chloride 5.0 g/l; HEPES 9 g/l; and 100 ml of heat activated porcine serum.

5. 2. 2. ORIGIN AND GROWTH OF M. MYCOIDES SUBSP. MYCOIDES SC USED FOR ANTIGEN PRODUCTION

The strain of *M. mycoides* subsp. *mycoides* SC used was B103 that was isolated from cattle lung in Portugal. Houshaymi *et al.* (1997) characterised it as a European strain by its ability to oxidise glucose but not glycerol. Cultures were initially grown in a 3 ml volume of medium before increasing stepwise to 10 ml, 100 ml and finally 1 litre. The inoculum size was one tenth of the medium volume. All cultures were incubated for 72 hours at 37°C in an atmosphere of 5 % CO_2 in air.

5. 2. 3. TEST ANIMAL SERA USED IN THE DEVELOPMENT OF THE LATEX AGGLUTINATION TEST

A positive control serum (ref CJ133) was produced at VLA (Weybridge) by inoculation of a cow with heat killed *M. mycoides* subsp. *mycoides* SC (strain Afadé). The inoculum at approximately 1 mg/ml was initially mixed 1:1 with Freund's complete adjuvant and subsequently with Freund's incomplete adjuvant. An Aberdeen Angus steer was inoculated with 1 ml of inoculum, sub-cutaneously across the rib cage at 6 weekly intervals, alternating between the left and right side of the ribs. Ideally the inoculum was given in four sites, however, if the animal was agitated this was reduced to three. A total of ten inoculations were given, and blood

samples were taken and tested by CFT 21 days post each inoculation. The maximum CFT titre of 1/40 was reached at 211 days post initial inoculation and was maintained until post-mortem at day 440.

A CBPP positive control serum (ref 845), from a naturally infected animal, was obtained from J. Regalla, LNIV, Lisbon. A standard negative serum and a positive *M. bovis* serum, used as controls for the routine *M. bovis* ELISA test, acted as negative controls.

In addition known CBPP negative field sera were obtained from British and Irish samples submitted routinely for *M. bovis* ELISA testing at VLA (Weybridge); Britain and Ireland have been free from CBPP for 100 years.

5. 2. 4. PREPARATION OF CARBOHYDRATE EXTRACT

The method of Rurangirwa *et al.* (1987b) was used with only minor modifications. In the first extraction, only the supernatant of a culture was used as these authors stated it to contain ten times more carbohydrate than cells; the supernatant from a one litre culture was adjusted to pH 5.0 with glacial acetic acid, boiled for one hour and filtered through Whatman paper. Two volumes of ethyl alcohol were added to the filtrate. This mixture was kept at 4°C overnight and the next day the precipitate was collected by centrifugation at 1000 *g* for 15 minutes. The precipitate was suspended in 20 ml distilled water (HPLC grade) and mixed at room temperature for 2 hours. The mixture was centrifuged at 3000 *g* for 30 minutes, the sediment discarded and the carbohydrate extracted by adding an equal volume of aqueous phenol (6 g phenol plus 1 ml of HPLC grade distilled water); the mixture was incubated for 1 hour at 68°C. After holding the mixture overnight at 4°C, it was centrifuged at 3000 *g* for 30 minutes. The aqueous layer was separated and run through a PD10 column (Pharmacia, Uppsala, Sweden) with HPLC grade distilled water (this replaced the dialysis steps used by Rurangirwa *et al.* (1987b). Two volumes of ethyl alcohol were added to the PD10 column void to precipitate the carbohydrate. The precipitate was mixed thoroughly, kept at 4°C overnight and then collected by centrifugation at 1000 *g* for 15 minutes. The precipitate was dissolved in 2 ml of HPLC grade distilled water, and run through the PD10 column as before. The precipitation and PD10 column step was repeated again before lyophilising the sample. The sample was resuspended in 2 ml of PBS (pH 7.2). To

separate the carbohydrates by size the sample was filtered through a column packed with BioSep Sec 3000, 60 x 2.1 cm, 230 ml volume (Phenomenex, UK), using an M45 HPLC/low pressure system (Waters, UK). The buffer used was PBS pH 7.2 with a flow rate of 4 ml/minute. From the first extraction 31 (4 ml) fractions were collected, pooled into five groups and concentrated by ethanol precipitation.

The following polysaccharide extractions were carried out: three from Eaton's medium following cell growth; one from cells grown in Eaton's medium; one from just Eaton's medium; one from PRM medium following cell growth; and one from cells grown in PRM medium. In these fractionations, the fractions were not pooled. In addition, where stated, the starting material for carbohydrate extraction was whole cells and not culture supernatant. Cells were separated by centrifugation from 1 litre of Eaton's broth after 72 hours incubation. The cells were washed twice and resuspended in 20 mls of PBS. Carbohydrates extracts were then carried out as described above.

5. 2. 5. BINDING OF POLYSACCHARIDE EXTRACT TO LATEX BEADS

Antigen was bound to latex beads (1.08μm, Sigma Chemicals, Poole, UK) by the method of Rurangirwa *et al.* (1987b). One ml of the latex beads (10% v/v suspension) were mixed with 1 mg of polysaccharide extract in 1 ml phosphate buffered saline, pH 7.4, containing 0.2 % sodium ethylene diamine tetra-acetate (Sigma Chemicals) and 0.01 % sodium azide (Sigma Chemicals). The mixture was shaken and incubated for one hour at 37°C and eight ml of the same buffer added.

Where stated, latex beads coated with antigen were incubated with proteinase K (Sigma Chemicals) at 20 mg/ml for 2 hours at 37°C.

5. 2. 6. SDS PAGE AND IMMUNOBLOTTING

Samples were run on a 12 % SDS PAGE using the standard technique of Laemmli (1970). The gels were stained using a Sigma silver staining kit exactly according to the manufacturer instructions. This method is more sensitive than Coomassie blue staining and stains both carbohydrates and proteins (Ochs *et al.*, 1981). Following SDS PAGE, gels were transferred to a nitrocellulose membrane using a semi-dry blotting system ('Transblot', Bio-Rad Laboratories, Hemel Hempstead, UK) for western blotting (Towbin *et al*, 1979). Control samples

containing molecular weight markers and a strip of both antigens were stained with the protein dye, amido black (Sigma Chemicals, Poole, UK) exactly following the manufacturer's instructions. The remaining membrane was cut into strips and blocked with PBS pH 7.2 containing 3 % w/v dried milk powder (Marvel) and 0.05 % v/v Tween 80 for 30 minutes at 37°C. The strips were then incubated with the positive CBPP antiserum, ref 845, for 60 minutes at 37°C; washed four times with 5 ml of PBS Tween; incubated with anti-bovine polyvalent immunoglobulin labelled with alkaline phosphatase conjugate for 60 minutes at 37°C (Sigma Chemicals, Poole, UK.); washed four times in PBS as before; and incubated at room temperature with 1 ml of the substrate 5 bromo-4-chloro-3-indoyl-phosphate/nitroblue tetrazolium (BCIP/NBT, Kirkegaard and Perry Laboratories, Maryland, USA). After sufficient colour had developed in the control samples, which took approximately 15 minutes the reaction was stopped by washing the strips in water.

5. 2. 7. ELISA OF CARBOHYDRATE EXTRACTS

The five pools obtained from the first carbohydrate extraction of culture supernatant were diluted 1 in 5 with carbonate/bicarbonate buffer (Sigma Chemicals) and 100 µl per well was used to coat a portion of a microtitre plate. Control wells were included that contained *M. mycoides* subsp. *mycoides* SC strain B103 whole cells, which had been washed three times in PBS, and were diluted to 1.5 µg/ml in carbonate/bicarbonate buffer. The microtitre plate was incubated at 4°C overnight. An indirect ELISA was performed with positive CBPP serum, negative CBPP serum and a potentially cross-reacting *M. bovis* positive/CBPP negative serum. Briefly, the coated plate was washed four times in a PBS solution containing 10 % milk powder and 0.5 % Tween 80. The sera were diluted 1/320 with the same buffer, and 100 µl quantities were placed in the appropriate wells and incubated for one hour at 37°C. The plate was washed as before, and 100 µl per well of a 1/4,000 dilution of IgG anti-cow horseradish peroxidase (DAKO, Denmark) specific conjugate were added. The plate was incubated for another hour at 37°C before washing and addition of 100 µl per well of 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Vetoquinol, UK). The reaction was stopped

when sufficient colour had developed (approximately OD_{450} 1.0) by adding 50 µl per well of 1 M citric acid. The specific OD_{450} for each well was measured using a plate reader (Labsystem, Basingstoke)

5. 2. 8. LATEX AGGLUTINATION TEST

Ten µl of latex beads with bound polysaccharide antigen were mixed with 10 µl of serum on a microscope slide and shaken for up to two minutes. Samples causing visible agglutination against a dark background were recorded as positive. Where stated sera were diluted 1 in 5 in PBS.

5. 2. 9. ANALYSIS OF CELL EXTRACTS

5. 2. 9. 1. TOTAL PROTEIN AND CARBOHYDRATE

Protein estimations on the carbohydrate extracts were carried out using a BCA protein estimation kit (Pierce, UK); this test is based on the Lowry method (Lowry *et al.*, 1951) and uses BSA as a standard. Carbohydrate estimations were made using the colorimetric phenol-sulphuric acid assay (Chaplin, 1994). Galactose was used to obtain a curve of absorbance (490 nm) against carbohydrate concentration.

5. 2. 9. 2. THIN LAYER CHROMATOGRAPHY

Thin layer chromatography (TLC) was performed using the method of Chaplin (1994). Control carbohydrates glucose, galactose, mannose, fucose, glucosamine and galactosamine; (Sigma Chemicals) were run with the test sample at a concentration of 8 μg. For the test sample the concentration had to be doubled. The test carbohydrate extracts were hydrolysed by suspending 5 mg in bijoux bottles containing one ml of 4N trifluoroacetic acid (Sigma Chemicals) and heated in an oven for two hours at 120°C. After cooling the samples were centrifuged at 3000 *g* for 20 minutes to free acid-insoluble residues from the suspension. The supernatant was then diluted with 4 ml of distilled water before being frozen and lyophilised and then resuspended, frozen and lyophilised again. The product was finally dissolved in 0.1 ml of distilled water for TLC analysis.

TLC analysis was carried out using Silica gel plates (Sigma Chemicals, UK). Samples were loaded 1.5 cm from the base of each plate at 1.5 cm intervals by repeatedly pipetting 2 μl volumes onto the plate and drying immediately with a hair drier so that dispersal was minimised. A total of 8 μl of the known sugar samples and 16 μl of hydrolysed extracts were loaded. Once dry, plates were placed in a equilibrated tank containing the buffer: butanol:pyridine:0.1M HCl in the ratio of 50:30:20 respectively. The TLC plates were run until the solvent front had migrated approximately 17 cm, which took approximately 4 hours. The plates were removed and allowed to dry in air before being developed. Plates were developed by spraying with either ninhydrin (Sigma Chemicals) or orcinol (Sigma Chemicals), following the manufacturer's instructions, allowed to dry in air before being heated at 110°C for 10 minutes to allow the colour to develop. Rf values were calculated using the Sigma guide and then adjusted to the $R_{glucose}$ value by dividing the Rf value by that obtained for glucose so that the Rf for glucose equals 1.0.

5. 2. 9. 3. QUANTITATIVE ANALYSIS OF CARBOIIYDRATES

Identification of the sugars present in cell carbohydrate extracts was carried out by BioMed Labs (Newcastle upon Tyne, UK), who specialise in glycoanalysis. Briefly, in their analysis monosaccharides were released by trifluoroacetic acid hydrolysis, separated using anion-exchange chromatography, and detected by a pulsed amperometric detection of the peaks in a Dionex Carbohydrate Analyser. The instrument was calibrated using a standard monosaccharide mixture.

Measurement of sialic acids was also undertaken on one sample. This sample was initially hydrolysed (by BioMed Labs) under milder conditions than required for neutral sugars. Following release of sialic acids, the concentration of trifluoroacetic acid was increased. Sialic acids were determined using the Dionex system, as described for neutral sugars, but with different chromatographic conditions. The reports are attached in Appendix 3.

Details of samples analysed by BioMed Labs are given in Table 5. 1.

TABLE 5. 1. DETAILS OF SAMPLES ANALYSED BY BIOMED LABS

Sample Number given by BioMed Labs	**Description**	**Analysis required**
98/01	2nd carbohydrate CBPP media extract not preserved with sodium azide	Monosaccharide
VL01	Carbohydrate extract from media only	Monosaccharide
VL02	Carbohydrate extract from cells	Monosaccharide
VL03	1st carbohydrate CBPP media extract preserved with sodium azide	Monosaccharide
VL04	2nd carbohydrate CBPP media extract	Sialic acid
Same as 98/01	not preserved with sodium azide	

5. 3. RESULTS

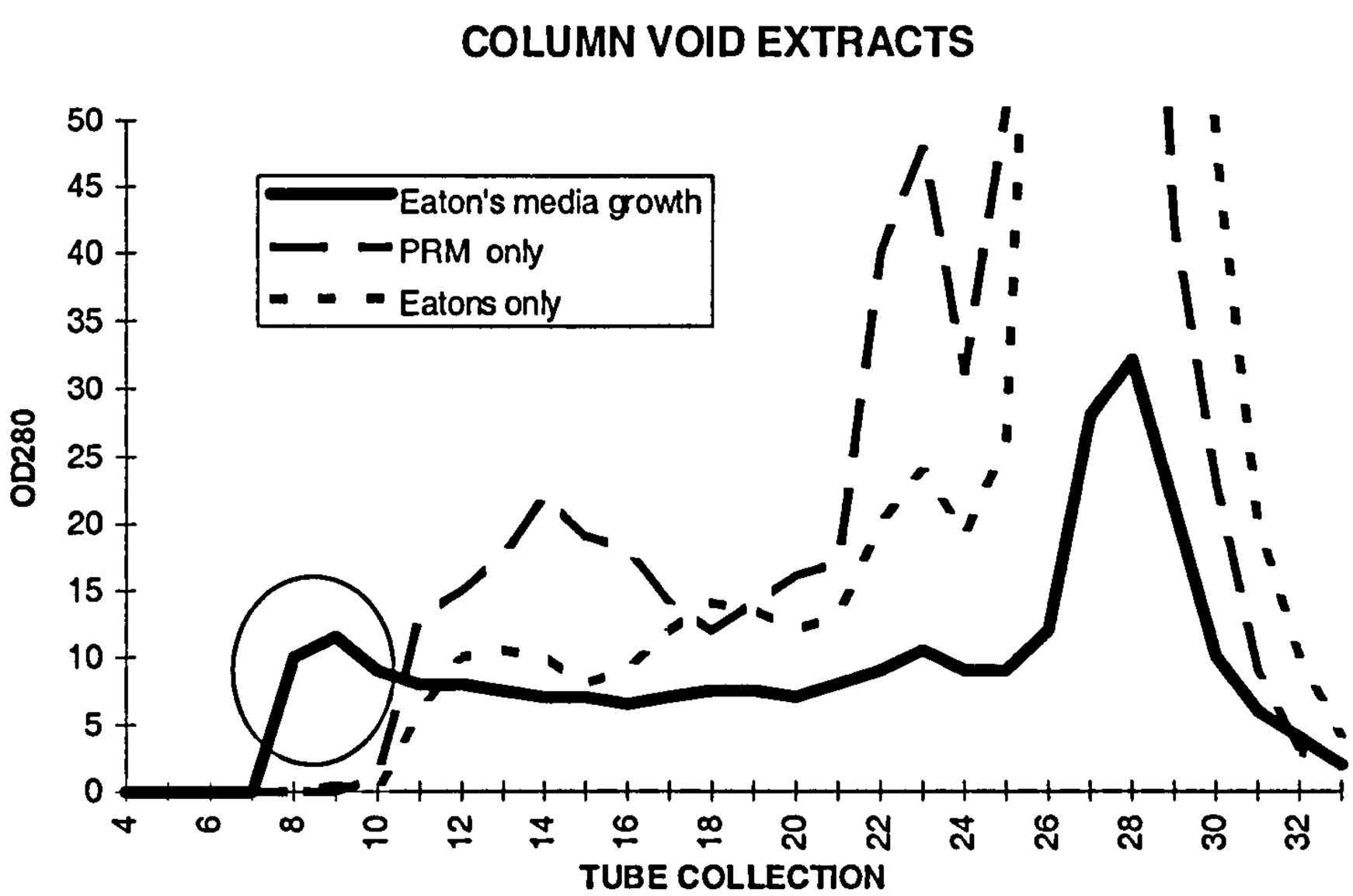
5. 3. 1. EXTRACTION AND FRACTIONATION OF CARBOHYDRATE ANTIGEN

Carbohydrate extracts were prepared from: the supernatant of cultures grown in Eaton's medium; Eaton's medium only; cells grown in Eaton's medium; the supernatant of cultures grown in PRM medium; PRM medium only; cells grown in PRM medium. Extracts were prepared and purified as described in methods (Section 5.2.4.). The aim was to identify the carbohydrates present in *M. mycoides* subsp. *mycoides* SC cells, and those secreted into the medium. Analysis of the medium had to be undertaken as medium contains carbohydrates.

5. 3. 1. 1. FRACTIONATION OF THE ANTIGEN FROM CULTURE SUPERNATANT

The carbohydrate extract from 1 litre of culture supernatant was applied to a Biosep Sec 3000 column and eluted with PBS pH 7.2 at a flow rate of 4 ml per second. Thirty-three fractions were collected. The OD_{280} of fractions is given in Figure 5. 1.

FIGURE 5. 1. THE ABSORBANCE OF FRACTIONS FOR A CARBOHYDRATE EXTRACT OF M. MYCOIDES SUBSP. MYCOIDES SC CULTURE SUPERNATANT COMPARED TO THOSE OBTAINED FROM EATON'S AND PRM MEDIUM ONLY



Graph shows OD_{280} readings for fractions collected from BioSep column. The samples shown compare fractions collected from Eaton's medium only, PRM medium only and Eaton's medium, which has grown *M. mycoides* subsp. *mycoides* SC cells. The circled area comprises the fractions included in Pool 1, which was used for the LAT.

The fractions were collected and pooled into five groups. Pool 1 is circled in figure 5. 1. This small peak from collection tubes 8 to 12 was present in all extracts from Eaton's medium which had been used to grow *M. mycoides* subsp. *mycoides* SC cells.

5. 3. 1. 2. FRACTIONATION OF THE ANTIGEN FROM WHOLE CELLS

Mycoplasma mycoides subsp. *mycoides* SC strain B103 was grown for 72 hours in both Eaton's medium and PRM medium. The carbohydrate was extracted as described in section 5.2.4. The column void extracts OD's are given in Figure 5. 2. and compared to that of the extract from Eaton's medium following growth.

FIGURE 5. 2. THE ABSORBANCE OF FRACTIONS FOLLOWING CARBOHYDRATE EXTRACT FROM CELLS COMPARED TO EATON'S MEDIUM FOLLOWING M. MYCOIDES *SUBSP.* MYCOIDES *SC GROWTH*

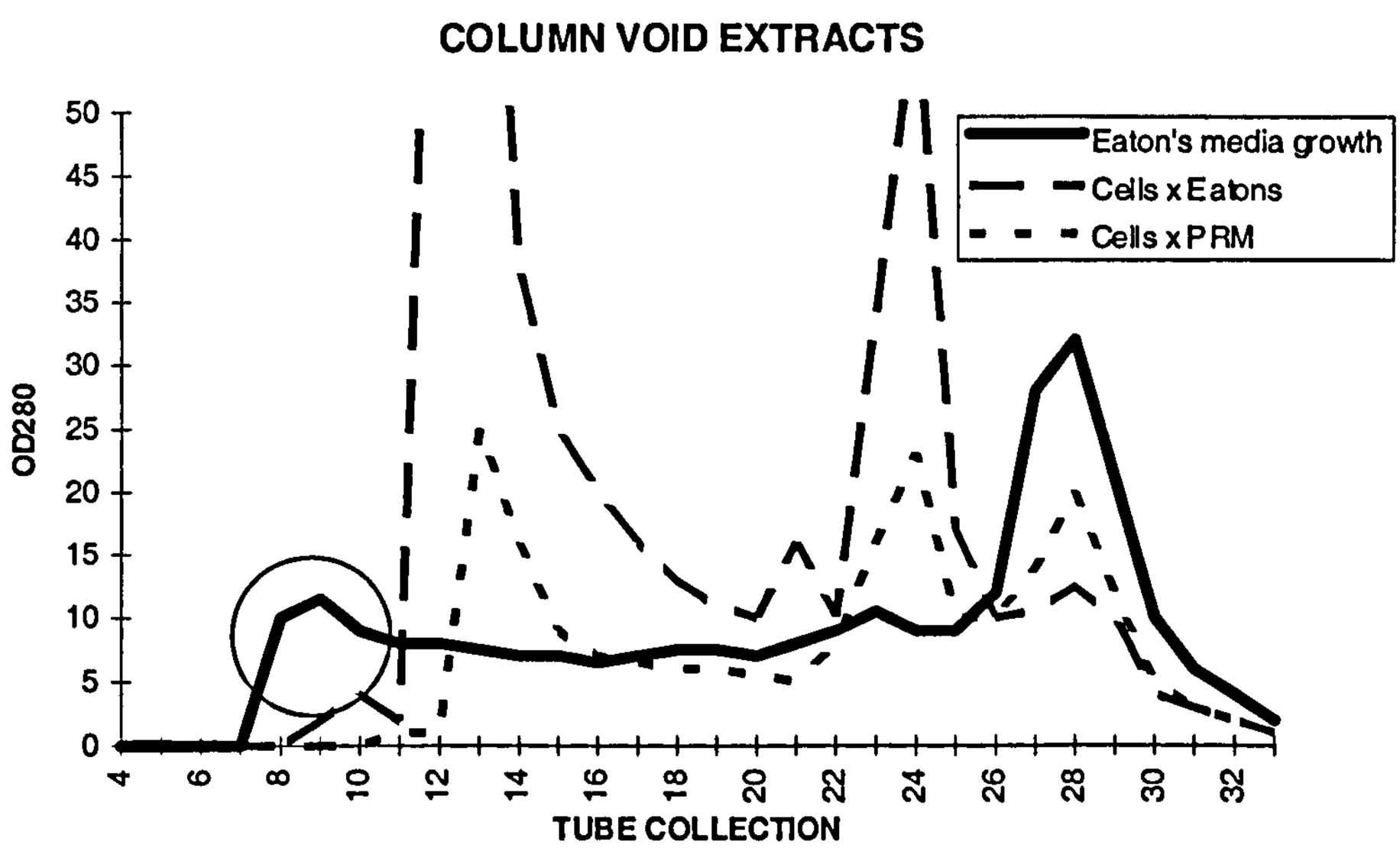


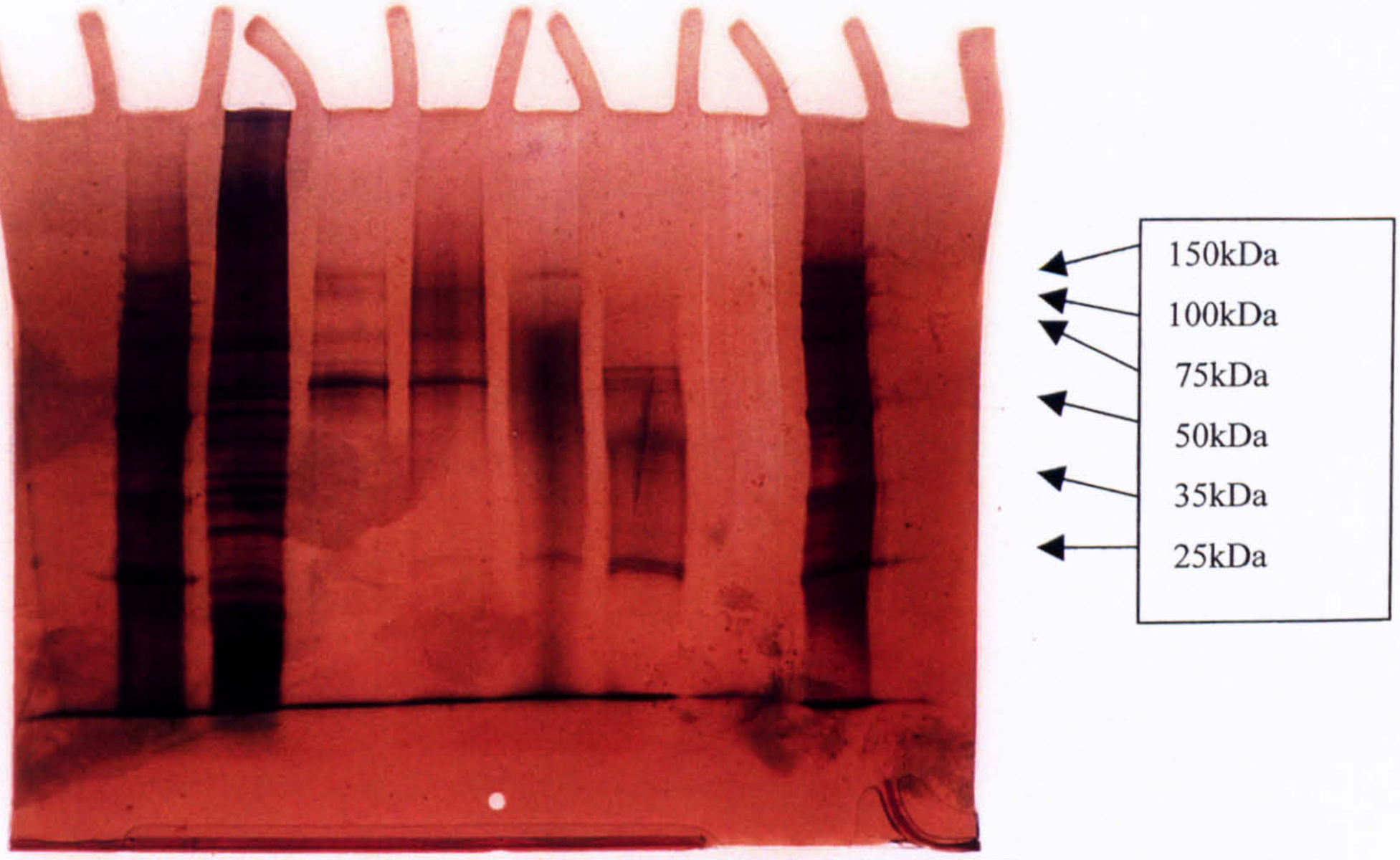
Figure 5.2. Graph shows OD_{280} readings for fractions collected from BioSep column which shows a clear difference in the fractions collected from carbohydrates extracted from cells and those collected from Eaton's medium which had supported *M. mycoides* subsp. *mycoides* SC growth. The fractions used for the LAT, tubes 8-12 are not present in cell extracts.

Eaton's medium on its own gave a similar profile without the early peak in the circled area. Extracts from cells grown in Eaton's or PRM medium gave large additional peaks over collection tubes 12 to 14 and an increased peak for collection tubes 22 to 25, but lacked the earlier peak at tubes 8 to 12.

5. 3. 2. PROTEIN CONTAMINATION OF CARBOHYDRATE EXTRACTS

The five pools obtained from the fractionation of the culture supernatants were assayed for protein content, which gave concentrations of less than 0.1 mg/ml. Sufficient SDS PAGE gels were run on these pooled fractions to provide duplicate gels for coomassie blue staining, silver staining and western blotting. These gels when stained, using a silver stain showed a small number of relatively faint bands compared to whole cell profiles. Pool 1 and 2 gave a similar band at approximately 65 kDa, and Pools 3 and 4 a double band at approximately 25 kDa; pool 5 gave no bands at all. Western blotting using CBPP positive serum (ref 845) gave no detectable bands. Following digestion with proteinase K, the protein content of individual pooled fractions were in all cases too small to detect, and no bands were visible on SDS PAGE gels stained with the silver stain (Sigma Chemicals, see section 5.2.6.).

FIGURE 5. 3. SDS PAGE OF POOLED EXTRACTS AND WHOLE CELLS



Lane 1 MWt; Lane 2, Whole *M. mycoides* subsp. *mycoides* SC cells (~10 µg); Lane 3, Pool 1; Lane 4, Pool 2; Lane 5, Pool 3; Lane 6, Pool 4; Lane 7, Pool 5; Lane 8, MWt.

5. 3. 3. IDENTIFICATION OF ANTIGEN IN CARBOHYDRATE FRACTIONS USING ELISA

The ELISA was used to determine if antigenic factions were present in the carbohydrate extracts. From the first extraction the ELISA results showed that all the pools gave some reaction with CBPP positive serum although none were as good as whole cells. All were negative with negative serum, but all reacted to some extent with *M. bovis* positive serum; for the cell extracts the biggest difference between ELISA results for control positive and negative sera was observed with Pool 1 (see Table 5. 2.). These results indicated it would probably be best to use Pool 1 for the development of the LAT. This was in accord with the evidence

obtained from the fractionation of cells and supernatants that showed that this pool was eluted with carbohydrates derived from cells rather than medium constituents.

TABLE 5. 2. DETERMINATION OF ANTIGENICITY IN POOLED CARBOHYDRATE EXTRACTS USING THE ELISA

	1. Positive* serum	2. Negative** serum	3. *M. bovis* +ve serum	Ratio of columns 1 : 3
Pool 1	0.41	0.06	0.31	1 : 0.76
Pool 2	0.30	0.02	0.25	1 : 0.83
Pool 3	0.26	0.02	0.21	1 : 0.81
Pool 4	0.14	0.00	0.21	1 : 1.50
Pool 5	0.16	0.03	0.29	1 : 1.81
Whole cells	1.02	0.10	0.44	1 : 0.43

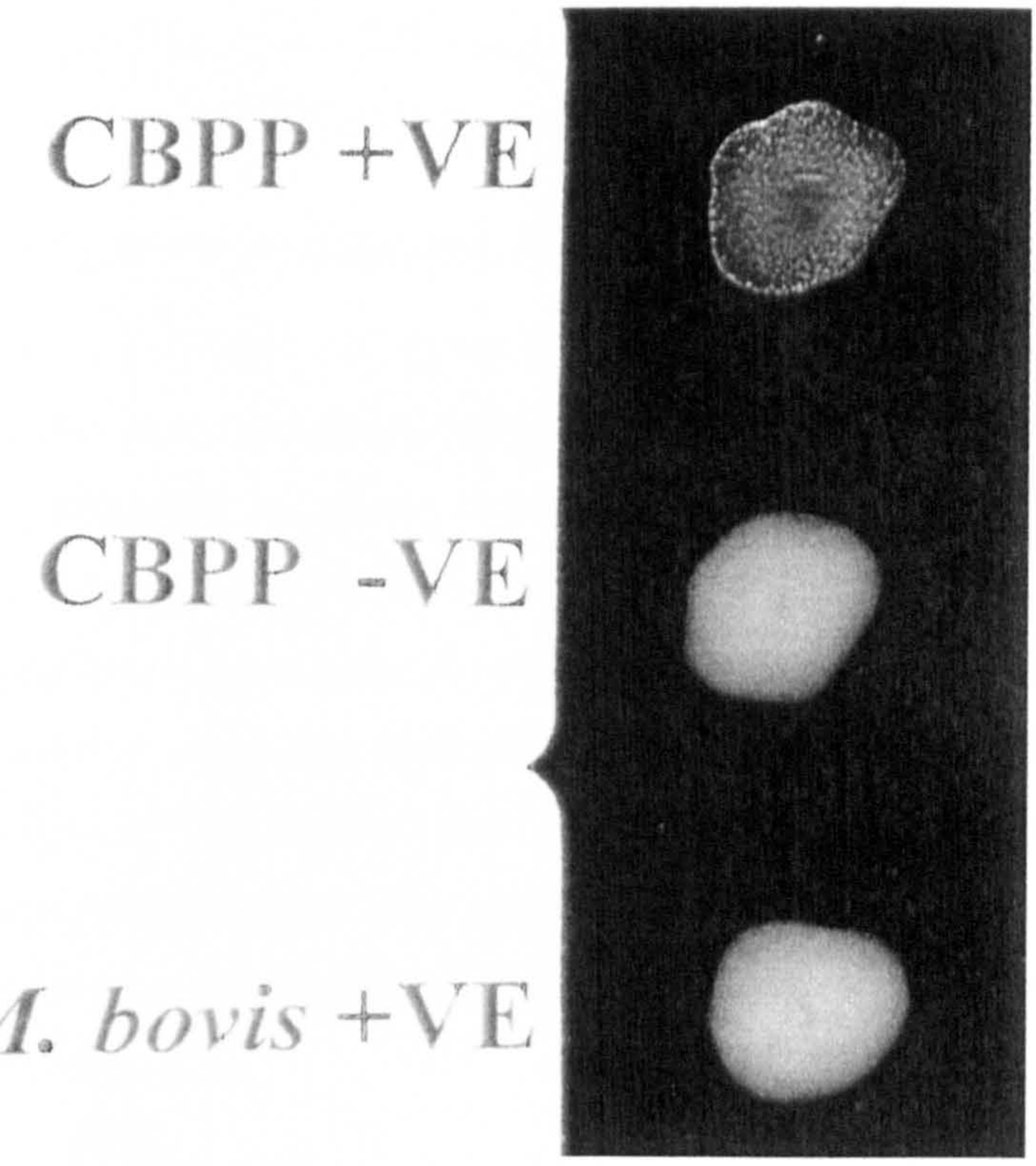
* CBPP positive serum from Portugal (ref: 845)

** Negative serum from Great Britain (ref: *M. bovis* ELISA negative control serum).

5. 3. 4. LATEX AGGLUTINATION TEST

Latex beads were coated with antigen (polysaccharide) as described in methods (section 5.2.5.). The antigen preparations used were all from Eaton's medium following *M. mycoides* subsp. *mycoides* SC growth. The first extract was used for all the evaluation work described in Chapter 6. The qualitative tests used CBPP positive, *M. bovis* positive and negative sera. Some cross-reactions were seen initially with some of the *M. bovis* positive field sera, but by diluting the bead concentration from 10 % to 1 % v/v, CBPP positive and negative sera were differentiated (Figure 5.4). However during the subsequent testing of 30 known negative sera, a batch of 20 sera from Ireland (CBPP free) gave 8 positive reactions. The sera remained positive after heat inactivation at 56°C for 30 minutes. By diluting 1 in 5 with PBS these sera became negative while positive controls remained positive. Therefore the test was modified to use a 1% v/v bead suspension with serum diluted 1 in 5 with PBS. Further evaluation of this test is described in Chapter 6.

FIGURE 5. 4. LATEX AGGLUTINATION TEST WITH CBPP AND M. BOVIS POSITIVE SERUM AND NEGATIVE SERUM



The figure shows a microscope slide (magnified approx. x 1.5) 2 minutes after the mixing of 1 in 5 diluted serum with 1 % v/v latex beads containing *M. mycoides* subsp. *mycoides* SC specific antigen. Agglutination is observed with serum from an animal with CBPP (Portugal, serum ref: 845). No reaction is seen with UK CBPP negative serum or of the serum from an animal with a *M. bovis* infection.

5. 3. 5. ANALYSIS OF CARBOHYDRATE EXTRACTS

The carbohydrate extract prepared from the supernatant of *M. mycoides* subsp. *mycoides* SC cultures from Eaton's medium was analysed for contaminating protein and carbohydrate components. The protein content was less than 0.1 mg/ml. The composition of the carbohydrate extract was investigated by TLC and by Dionex carbohydrate analysis. TLC suggested the presence of three sugars that ran at $R_{glucose}$ values of: 1.10, 0.77, 0.55 (see Table 5.3).

5. 3. 5. 1. ANALYSIS OF EXTRACTS: TLC

TLC was carried out on the pooled carbohydrate extracts (pool 1) four times in all. Plates were developed using ninhydrin or orcinol. With the ninhydrin stain, all spots were blue in colour, however the test sample and the glucosamine were pale in comparison to the other samples. With the orcinol stain, most of the spots were a pinkish colour, with the glucosamine being more intense, red. The colour differentiation expected with the orcinol stain, as described by Chaplin (1994), where glucose was stained violet; galactose and mannose, blue; fucose pink; glucosamine and galactosamine, grey was not observed.

The $R_{glucose}$ values were calculated for the known and unknown samples and is given in Table 5. 3.

TABLE 5. 3. TLC ANALYSIS OF HYDROLYSED CARBOHYDRATE EXTRACTS FROM CULTURE SUPERNATANTS

Carbohydrate	Mean $R_{glucose}$ values calculated from duplicate TLC plates Actual values in brackets (i.e. adjusted for Glucose = 1)	$R_{glucose}$ values (Chaplin, 1994)
Glucose	1.00	1.00
Mannose	1.07 (1.07; 1.07)	1.13
Galactose	0.95 (0.94, 0.95)	0.89
Fucose	1.12 (1.11; 1.12)	1.34
Glucosamine	0.75 (0.70, 0.80)	0.72
Galactosamine	ND	0.55
Carbohydrate extract	1.10, 0.77, 0.55 (1.09, 1.11; 0.76, 0.77; 0.54, 0.55)	ND

The TLC and calculated $R_{glucose}$ values indicated that three carbohydrates were present in the carbohydrate extract. The values obtained indicated that mannose or fucose may be present as $R_{glucose}$ values obtained for them were 1.07 and 1.12 respectively compared with 1.10 for the test sample. A second test value of 0.77 compared with glucosamine at 0.75 and the third value of 0.55 did not match with any controls on this plate. Comparison with data obtained by Chaplin (1994) using the same method would suggest that galactosamine may also be present.

5. 3. 5. 2. ANALYSIS OF EXTRACTS: DIONEX CARBOHYDRATE ANALYSER

Dionex carbohydrate analysis was performed (by BioMed Labs) on four carbohydrate extracts:

(i). an extract from the supernatant of a culture grown in Eaton's medium

(ii). an extract from the supernatant of a culture grown in Eaton's medium, but containing the preservative sodium azide.

(iii). a cell extract from cells grown in Eaton's medium.

(iv). an extract from uninoculated Eaton's medium.

Sialic acid analysis was also performed (by BioMed Labs) on the supernatant of a culture grown in Eaton's medium, sample (i) above.

Six different sugars were detected in one of the carbohydrate extract samples (see Table 5.4). These were: fucose, galactose, glucose, N-acetyl-galactosamine, N-acetyl-glucosamine and mannose. Importantly, the uninoculated medium contained carbohydrate, with the predominant sugars being: N-acetyl-galactosamine and mannose (29.0 and 34.1 % respectively). The extract from whole cells contained predominantly galactose. This was expected as galactose has been reported to be the major sugar >90 % (Plackett and Buttery, 1964) of the cell capsule. Glucose and fucose were also detected at 13.0 and 10.0 % of total sugars respectively. Glucose has been reported to contribute up to 10 % of the mass of the capsule of *M. mycoides* subsp. *mycoides* SC strain V5. However the finding of fucose was unexpected. Some fucose may be expected to be present due to contamination with carbohydrate material present in the medium; however, the % of fucose in the whole cell extract (10.0 %) was greater than that for the extract from the fraction from uninoculated medium (6.8 %). The low levels of N-acetyl-glucosamine and mannose (1.6 and 1.9 %) detected in the cell extract are, however, probably due to contamination from medium carbohydrates.

The extracts from the supernatant of a culture grown in Eaton's medium contained predominantly galactose (83.2 %). N-acetyl-galactosamine and fucose were also present at 11.4 and 5.4 % respectively. These may be contaminants from the medium as they were found to be present at 14.7 and 6.8 % respectively in the uninoculated Eaton's medium; N-acetyl-galactosamine was not detected in the cell

extract. The other sample from the supernatant of a culture grown in Eaton's medium was preserved in sodium azide, which as a heavy metal may affect analysis by the Dionex carbohydrate analyser; it also contained predominantly galactose (59.5 %), at a lower level that that obtained without sodium azide in the sample. N-acetyl-galactosamine and fucose was also present at 9.0 and 6.2 % respectively, which is at similar levels to that detected in the previous sample. This sample also contained N-acetyl-glucosamine and glucose at 21.4 and 3.8 % respectively, which are at similar levels to that found in the uninoculated Eaton's medium. However, no mannose was detected which was present at 34.1 % in the uninoculated Eaton's medium.

Analysis of the supernatant of a culture grown in Eaton's medium for sialic acid showed that N-acetyl neuraminic acid was present. Another unidentified peak was also detected. Copies of the full reports from BioMed Labs are given in appendix 3.

In summary, samples from *M. mycoides* subsp. *mycoides* SC cells and from medium that had supported cell growth showed elevated levels of galactose, as expected. The levels of galactose were shown to be as high as 83.2 %. These results also showed that fucose was present. Fucose is a medium component, but it was also present in all samples and accounted for 10 % of the total sample from the cell extracts. If it were not a component of the cell, then its content would be much lower on the cell extract, rather than higher.

TABLE 5. 4. RESULTS OF SAMPLES ANALYSED BY DIONEX CARBOHYDRATE ANALYSER

Source of extract	Carbohydrate	Percentage	nmol/mg
Carbohydrate extract from unininoculated Eaton's medium	Fucose	6.8%	30.3
	N-acetyl-galactosamine	14.7%	65.1
	Galactose	11.7%	51.8
	Glucose	3.7%	16.4
	N-acetyl-glucosamine	29.0%	128.8
	Mannose	34.1%	151.3
Carbohydrate extract from cells grown in Eaton's medium	Fucose	10.0%	4.3
	N-acetyl-galactosamine	ND	ND
	Galactose	73.5%	31.7
	Glucose	13.0%	5.6
	N-acetyl-glucosamine	1.6%	0.7
	Mannose	1.9%	0.8
Carbohydrate extract from culture supernatant fractions 8-12	Fucose	5.4%	28.7
	N-acetyl-galactosamine	11.4%	60.3
	Galactose	83.2%	441.9
Carbohydrate extract from culture supernatant preserved with sodium azide fractions 8-12	Fucose	6.2%	1.3
	N-acetyl-galactosamine	9.0%	1.9
	Galactose	59.5%	12.5
	Glucose	3.8%	0.8
	N-acetyl-glucosamine	21.4%	4.5
	Mannose	ND	ND
Carbohydrate extract from culture supernatant fractions 8-12	Sialic acid present as:		
	N-acetyl neuraminic acid		76.8

5. 4. DISCUSSION

The aim of the work presented in this chapter was to isolate and identify the polysaccharide antigen(s) produced by *M. mycoides* subsp. *mycoides* SC and to establish whether the antigen could be used in the development of a latex agglutination test (LAT) for CBPP. It is known that *M. mycoides* subsp. *mycoides* SC strains produce large quantities of extracellular polysaccharides and this approach has previously been used successfully in the development of a LAT for CCPP. The nature of any polysaccharide isolated was also of interest as the capsular polysaccharide of *M. mycoides* subsp. *mycoides* SC has been associated with pathogenicity and there is some controversy as to its composition.

5. 4. 1. POLYSACCHARIDE MATERIAL PRODUCED BY M. MYCOIDES SUBSP. MYCOIDES SC

The role of the capsule in disease is of obvious interest. Smith (1984) stated that there appears to be no correlation between the presence of a capsule and pathogenicity even though the mycoplasmas having capsules are all pathogenic. However, capsules are generally considered to contribute to pathogenicity by promoting binding to host tissue surfaces and enhancing resistance to phagocytosis. In addition, there is some evidence that the capsule of *M. mycoides* subsp *mycoides* SC might have a direct toxic effect on host cells and its structural similarity to the host pneumogalactan further suggests that it might induce auto-immune reactions (Hudson, 1971). Interestingly, mycoplasma capsules usually only comprise 1 to 3 % cellular dry weight while *M. mycoides* subsp. *mycoides* SC comprises 10 %.

The presence of a specific polysaccharide obtained from *M. mycoides* subsp. *mycoides* SC was described by Buttery and Plackett (1960). The earlier conflicting reports of Kurotchkin, (1937), Dafaala, (1957, 1959), White (1958) and Knight and Cowan (1961), where various antigenic properties were reported, may be due to different extracts being examined. From the work in this study, initially using the ELISA method, it was found that polysaccharides have antigenic activity, with the minor peak seen in Figure 5. 1. being the most antigenic. Other workers had investigated different factions that were subsequently extracted from the whole column void. Comparisons of void extracts from medium, medium extract and cells

showed that the major peak, seen in tubes 28 – 31, was also present in all samples including the medium only sample. The cells produced a much larger peak in tubes 12-14 that is probably attributed to galactose extracted from the capsule. Although this may be a major antigenic component, more evaluation is necessary for its use in serological tests.

The presence of a sialic acid, *N*-acetyl neuraminic acid is also noteworthy, possibly supporting the findings of Yoshida (1961) who reported that lipid factions contained complement-fixing antigens. Although, Smith (1984) found that deacylation of glycolipids does not alter their reactivity with specific antibody, thus the lipid portion of the molecule is not thought to be involved in antigen/antibody reactions.

More recently March *et al.*, (1999) described the polysaccharide as having a complex composition, comprising of mannose, glucose, galactose, fucose, glucosamine, and galactosamine. In this study these components were obtained from the analysis of uninoculated medium, suggesting that the samples that March *et al.*, (1999) analysed were contaminated with medium. Waite and March (2001), reported that most of the high molecular weight carbohydrates capsular polysaccharide extracts could be attributed to BactoTryptose in the culture medium, but suggested that galactose may be a major capsular polysaccharide component. In an attempt to confirm this finding using lectins, Waite and March, (2001) failed to identify the carbohydrate, as it only reacted weakly with lectins and concluded that the capsular polysaccharide must be present in an unusual configuration. This emphasises the need for care in extraction of the capsular polysaccharide and analysis of the data obtained.

Electron microscopy studies on *M. mycoides* subsp. *mycoides* SC preparations stained with ruthenium red (Minion and Rosenbusch, 1993) provided evidence that these rudimentary capsules were composed of polysaccharides. Rurangirwa *et al.* (1987a) found that the polysaccharide of *M. capricolum* subsp. *capripneumoniae* was also extractable from the supernatant of culture medium following cell growth. Whether, this is actively excreted from the cell or part of the natural capsule breakdown, is not known. However, the capsular polysaccharide is

found in large quantities in the medium, which may indicate that the polysaccharide is actively excreted.

The methods described for purification of the polysaccharide from either cells or medium supernatant uses very similar approaches. The difficulty arises in removing or recognising that 'contaminating' material may be present either from the culture medium or other components of the cells. In this study only a fraction of the column void was used, thus avoiding the main medium components.

Despite only using a fraction of the column void, when the sample was analysed by SDS PAGE, it was shown by the sensitive silver-staining method that some proteins were present in the extract, although these were not readily detectable by protein estimation methods. The proteins were removed by proteinase K digestion and were no longer visible by SDS PAGE, but the *M. mycoides* subsp. *mycoides* SC extract was still antigenic as measured by the ELISA and LAT.

5. 4. 2. COMPOSITION AND PURITY OF THE POLYSACCHARIDE FROM M. MYCOIDES SUBSP. MYCOIDES SC

As described in section 5.4.1. previous workers have reported different findings on the composition of the capsular polysaccharide. Plackett and Buttery, (1958) described it as being 90% galactose and 10% glucose, whereas March *et al.*, (1999) found it comprised of mannose, glucose, galactose, fucose, glucosamine, and galactosamine. Interestingly, reports on other closely related organisms such as *M. mycoides* subsp. *capri* described it as being mainly glucose with a small amount of galactose (Jones *et al.*,1965), and Rurangirwa *et al.* (1987a), working on *M. capricolum* subsp. *capripneumoniae* (F38), reported approximately equal amounts of glucose, galactose, mannose, fucose, galactosamine and glucosamine. When examining the column void graph (figure 5.2.) whole cells give a different profile to that of medium and also medium supernatant. It appeared from the ELISA and SDS PAGE results that Pool 1, which contained a small peak observed only in supernatant extracts of the Eaton's medium, was the most effective in the LAT. Therefore the component of this peak was of specific interest and worthy of further investigation. It is probable that the main polysaccharide composition of cells is in column void tubes 12-15, which would help explain the conflicting reports and the problem with medium component 'contamination'.

The main polysaccharide obtained in this study was galactose, which is broadly in agreement with the findings of Plackett and Buttery (1958). In this study the amount of galactose detected was lower, at 83.2 % from culture supernatant, 73.5 % from cells and only 59.5 % (sodium azide treated extract). Glucose was detected in the medium and the sodium azide treated extract at 3.7 % and 3.8 % respectively, which rose to 13.0 % from the cell extract, suggesting glucose is present in the cells, but not excreted into the medium. The presence of fucose was not expected, although it is present in the medium-only extract at 6.8 %. The detection of similar levels in the culture supernatant, may be accounted for by medium contamination, but the higher level of 10 % in the cell extract would indicate that fucose is one of the carbohydrates present in the cell.

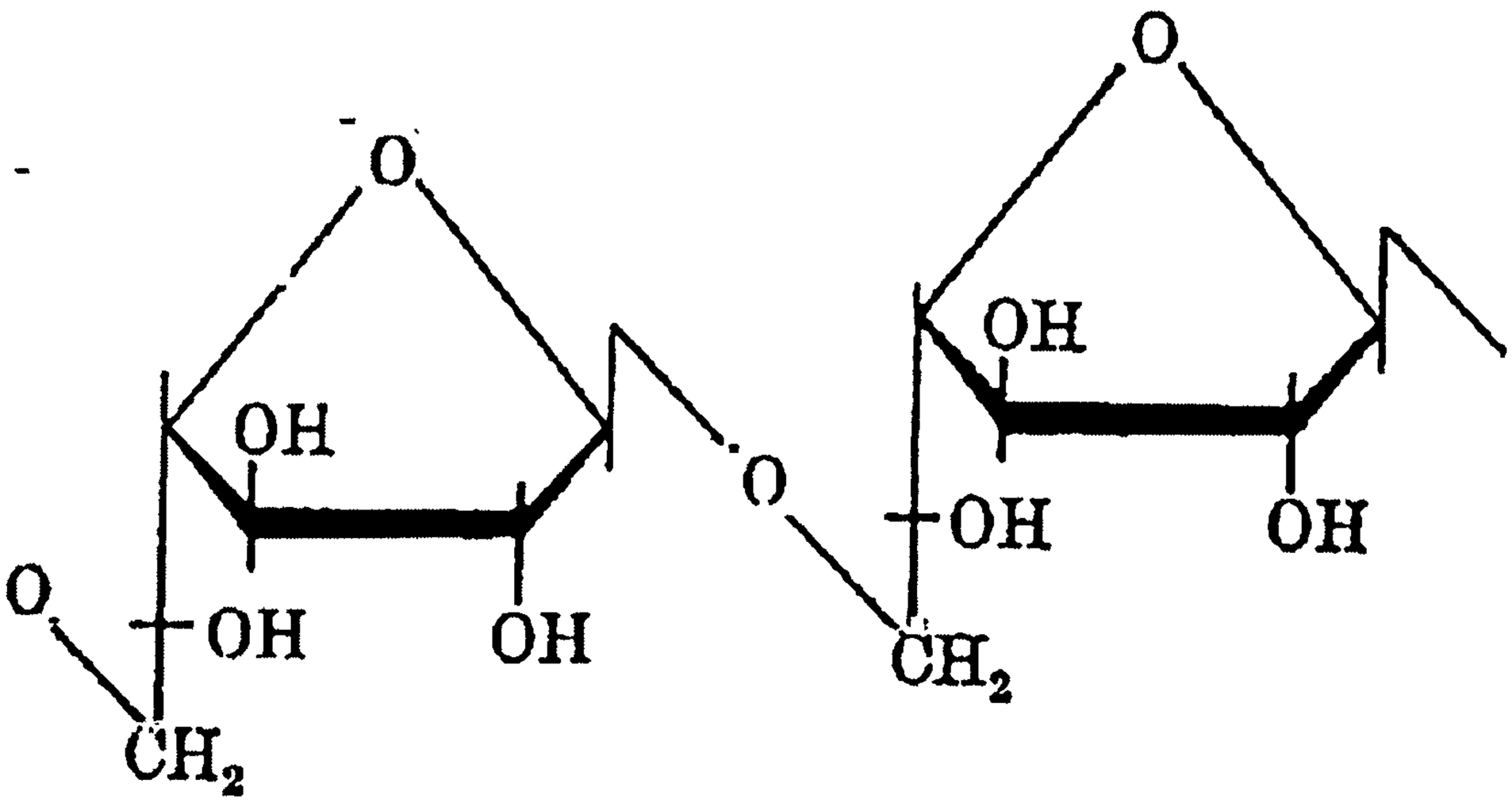
The TLC clearly showed the presence of three components in the carbohydrate extract. However, this test was limited as identification of the components was not conclusive. The presence of more than one carbohydrate in the test sample may affect migration rates. One of the other major drawbacks is that TLC was not quantifiable. The use of Dionex carbohydrate analysis confirmed the presence of three major components but also quantified those components.

Specific lectins could be used to identify carbohydrates as indicated by Smith (1984), however previous experience has shown that the quality and specificity of lectins varies from supplier to supplier (Gill, K. personal communication). Interestingly, Waite and March (2001) tested capsular polysaccharide material from *M. mycoides* subsp. *mycoides* SC and obtained weak reactions with lectins, specific for *N*-acetylglucosamine dimers and trimers, (α-1,3) and (α-1,6) mannose, terminal α-/β- *N*-acetyl glucosamine (galactose) and galactose, galactosyl (β1-4) *N*-acetylglucosamine.

The component analysis of the antigen agrees with the findings of Plackett *et al.* (1963). It would be interesting to analyse the structure of the polysaccharide, although it is assumed to be similar to that reported by Plackett and Buttery (1964) which is shown in figure 5.5. It may be worth pursuing in future, as well as investigating the use of synthetic polysaccharides. Previous workers (Buttery and Plackett, 1960) discovered glycolipids were present in *M. mycoides*. In this thesis N-acetyl neuraminic acid (a sialic acid) was identified, a sugar unit often associated with glycoproteins and glycolipids. One of the

difficulties in preparing antigens and their analysis is the potential for batch to batch variation. The potential for variation was minimised by standardising culture incubation times and temperatures and also by following the same procedure for carbohydrate extraction and analysis.

FIGURE 5. 5. MOLECULAR STRUCTURE OF GALACTAN



Molecular structure of the galactofuranosyl disaccharide repeating unit of galactan from *Mycoplasma mycoides.*
Plackett and Buttery (1964).

5. 4. 3. THE LATEX AGGLUTINATION TEST

The extract from 'spent' medium produced carbohydrates that are antigenic to *M. mycoides* subsp. *mycoides* SC as measured by the ELISA and LAT. The extraction method used in this thesis produces antigenic components that appear to be specific for detection of CBPP antibodies using the LAT. The quantities and methods used here followed the guidelines of Rurangirwa (1995), but still required fine-tuning. The initial difficulties associated with the spontaneous agglutination of

the beads and the non-specific interactions were overcome by manipulation of the concentrations of beads, antigens and serum. Standardising production methods so that specific concentrations of the components and the coating of latex beads to give consistently reproducible tests is paramount.

In addition, Mab production using the antigenic components of the extract may enable development of novel whole cell (antigen) detection systems. Such a Mab could be bound to latex beads to produce a detection system for antigen in nasal exudates or lungs at post-mortem, or as a rapid confirmatory identification test for *M. mycoides* subsp. *mycoides* SC cultures in the laboratory.

The LAT results, using Pool 1 (extracts), were accurate and generally in agreement with CFT and ELISA results. If these results can be reproduced on future extracts it will be worth testing with whole blood, instead of serum. Field trials could then be carried out with collaborators in Portugal and Uganda. From the preliminary data obtained in this study it was decided to evaluate the LAT test further, testing more serum. This work is described in chapter 6.

CHAPTER 6

6. 1. CRITICAL EVALUATION OF THE CBPP LATEX AGGLUTINATION TEST AND COMPARISON WITH OTHER SEROLOGICAL TESTS

6. 1. 1. INTRODUCTION

The aim of the work reported in this chapter was to evaluate the newly developed latex agglutination test (LAT) (see chapter 5) in comparison with other serological tests. The LAT was compared to existing testing procedures; these were: the complement fixation test (CFT) (Campbell and Turner, 1953); immunoblotting (Nicholas *et al.*, 1996), the competition (c) ELISA (Le Goff and Thiaucourt (1998); which was carried out as part of a comparative trial arranged during a workshop of an EC COST Action (COST Action 826 on Mycoplasmas of Ruminants); and a newly developed ELISA based on tween 20 treated *M. mycoides* subsp. *mycoides* SC cells. Details of gross pathology of the affected cattle was available for some samples. These tests were all carried out on serum samples. Obtaining well-defined and characterised sera is one of the difficulties associated with evaluating tests. In this study, several sources of sera were obtained and are described in Section 6. 2. 1.

The CFT is the OIE recommended serological test despite giving false positive results and only detecting approximately 70 % of CBPP affected animals. Although tests such as the cELISA and immunoblotting have been developed there is no "gold standard" serological test. Comparisons with gross pathology are not always possible, and carrier animals and those in the early stages of infection may go undetected.

6. 2. MATERIALS AND METHODS

6. 2. 1 SOURCES OF ANIMAL SERA

6. 2. 1. 1. SERA FROM AN EXPERIMENTAL INFECTION

An experimental infection was carried out as part of the EC FAIR programme CT95-0711 in Italy; this provided both whole blood and sera throughout the experiment giving well defined material for examination. Friesian cattle (18 –

36 months) were screened to exclude the presence of: endoparasites; antibodies to *M. mycoides* subsp. *mycoides* SC; brucellosis; tuberculosis; leucosis; and IBR, BVD/MD and PI3 viruses. Culture and PCR of nasal swabs also confirmed the absence of the *M. mycoides* cluster including *M. mycoides* subsp. *mycoides* SC. Three animals were intubated with 10 ml of $10^{8.87}$ organisms per ml of *M. mycoides* subsp. *mycoides* SC strain B345/93, originally isolated from pleural fluid in Portugal. After one-month a fourth cow was placed in contact with the other three. Cow 1 and cow 4 were re-intubated 19 weeks after the start of the experiment.

The animals were clinically examined daily. Blood and nasal swabs were taken weekly and sent to VLA (Weybridge). After 21 weeks cows 2 and 3 were slaughtered, with cows 1 and 4 being slaughtered at 26 weeks. Post-mortem examinations were conducted and blood, nasal mucosa, tonsils, lung, mediastinal-cranial lymph nodes, tracheal scrub, spleen and kidney were collected for testing.

The weekly serum samples from the four cattle were tested by whole cell ELISA, tween 20 treated ELISA and by the LAT (see section 6.3.). Some of the whole blood was also tested by the LAT.

6. 2. 1. 2. CONTROL NEGATIVE SERA

Sera were selected randomly from samples submitted to the VLA (Weybridge) for serological testing for *M. bovis* infection. In excess of 250 sera were tested. The UK has been free of CBPP for over one hundred years.

6. 2. 1. 3. SERA FROM CBPP AFFECTED FARMS IN PORTUGAL

Sera from 169 cattle were collected from six herds in which CBPP was suspected. The sera were kindly supplied by Dr. J. Regalla, (LNIV, Lisbon, Portugal) and had previously been tested by CFT and immunoblotting. Post mortem data was also available. The LAT and ELISA were carried out 'blind' and the results were compared with previous tests only after testing was completed.

6. 2. 1. 4. OTHER SERA

Other serum samples tested were: eight CFT positives from Portugal (see chapter 4); seventeen CFT positive sera obtained from the Italian outbreaks in 1993; and thirty-one sera obtained from clinically affected herds in Uganda.

A panel of 134 sera containing: CFT positive sera from Portugal which were negative for CBPP by immunoblotting; known positive sera supplied by CIRAD-EMVT, France; and 58 control negative sera from the UK. The sera were distributed by CIRAD-EMVT, France as part of an EC COST Action 826 testing programme. The serum samples were coded and the code was only revealed after testing.

Two hundred and twenty five sera from cattle in Hungary with pneumonic disease were also tested by LAT and the tween 20 ELISA.

6. 3. SEROLOGICAL METHODS

The LAT was conducted on all serum samples as described in Chapter 5 (section 5.2.8.). The coated beads used were from a single batch of carbohydrate extract (1st extract reference VL03 in Table 5.1.).

The whole cell ELISA was carried out as previously described (sections 5.2.7.) on serum samples from Portugal.

A modified whole cell ELISA was also used where stated. A tween 20 extracted antigen was produced as follows: tween 20 solubilisation was carried out essentially according to Nicolet *et al.* (1980). Cells were harvested by centrifugation at 10,000 *g* for 40 min at 4°C, the supernatant discarded and the cell pellet resuspended in 0.0125M PBS containing 1 % tween 20 (1 ml of buffer per mg of protein). Cells were incubated for two hours at 37°C with constant agitation. The cell fraction was separated from the soluble fraction by centrifugation at 10,000 *g* for 40 min at 4°C. The pellet was washed once in 0.1M PBS and resuspended in a final volume of 20 ml. The soluble fraction was filtered through a 0.2 μm pore diameter filter prior to PD-10, Sephadex-25 (Pharmacia) column purification. The surface antigens that were soluble in Tween 20 were found to be one of the major cross-reacting components with *M. bovis,* so the remaining component of the whole cells was used as an antigen and was standardised by chequer-board titration. The ELISA procedure was essentially the same as that described for the whole cell ELISA (see Section 5.2.7.).

Immunoblotting was carried out on samples from Italy and Portugal (see chapter 4). The method was the same as described by (Nicholas *et al.*, 1996), using Portuguese strain B103 as the antigen.

Other serological data included in the results section were obtained from the serological testing section at VLA (Weybridge), or at collaborating laboratories in Europe. These tests were carried out as described by Campbell and Turner, (1953) CFT; Tittarelli *et al.* (1999) modified CFT; Le Goff and Thiaucourt, (1998) cELISA; Gonçalves *et al.* (1998) immunoblotting. Data obtained by other researchers is acknowledged in all cases.

6. 4. STATISTICAL ANALYSIS

Determination of the correlation of the results obtaioned by the LAT with those of other serological tests was based on kappa coefficients (Cohen, 1960). Kappa is determined as

$$\frac{\text{Expected agreement - Random agreement}}{\text{Maximum expected agreement - Random agreement}}$$

Kappa is 0 if there is no evidence of agreement between tests. A value of 1 is obtained if there is perfect agreement. Standard errors for kappa were determined. The statistical unit at VLA (Weybridge) performed the statistical analyses on the data given in Table 6.2.

6. 5. RESULTS

6. 5. 1. SERUM SAMPLES TESTED BY LAT

The serum samples tested by the LAT and the results of the tests are summarised in Table 6. 1. The samples from Hungary and the UK gave no clear positive agglutination, although two of 250 UK samples gave slight agglutination. One of the samples was turbid, due to growth of a contaminating micro-organism, and this may have affected the result due to cloudiness. Neither of the two UK samples that gave agglutination were positive in CFT or by immunoblotting. Since the UK and Hungary have been free of CBPP for many years, positives were not expected, and thus the number of false positives in European cattle would appear to

be less than 0.5 %. For the Hungarian serum samples the negative LAT results were confirmed by the tween 20 whole cell ELISA test; all samples were negative.

The batches of serum samples obtained from Uganda, Italy and Portugal all contained samples that were positive by the LAT. The samples obtained from the Italian cattle (17 in total) gave seven LAT positive results, four were positive by CFT and five were positive by immunoblotting. All samples that were positive by CFT were also positive by immunoblotting, and all samples that were positive by immunoblotting were also positive by LAT.

The samples obtained from Uganda (31 sera) gave 18 LAT and CFT positive results and whole cell ELISA gave 16 positive results with 7 samples being in the undetermined/suspect range, (see Table 6.1.). However, not all tests that were positive by CFT were positive by ELISA or LAT and vice versa. Agreement was calculated as similar test/total tests X 100 %. There was an 74 % agreement between the CFT and the LAT and a 75 % agreement between the ELISA and the LAT if the ELISA results in the suspect range are ignored, if those results are taken as positive then there is a 71 % agreement, or 68 % if taken as negative.

TABLE 6. 1. RESULTS OF SEROLOGICAL TESTS ON SERA OBTAINED FROM UGANDA

Sample Number	CFT (VLA) Titre	ELISA (Whole cell) OD	LAT
1	**1:160 ++**	**1.40**	**+ve**
2	**1:160 ++**	**0.36**	**+ve**
3	**1:20 ++**	**0.97**	**+ve**
4	-ve	0.23	-ve
5	**1:40 ++**	0.26	**+ve**
6	**1:20 ++**	**0.48**	-ve
7	**1: 40 +++**	0.28	**+ve**
8	**1:160 +++**	**0.72**	**+ve**
9	**1:160 ++**	0.28	-ve
10	-ve	0.19	-ve
11	**1:160 ++**	**0.75**	**+ve**
12	**1:20 +++**	**0.95**	**+ve**
13	-ve	0.25	-ve
14	**160 +++**	**0.68**	**+ve**
15	**1:20 +++**	0.22	**+ve**
16	-ve	0.18	-ve
17	-ve	0.22	**+ve**
18	-ve	0.14	-ve
19	**1:640 ++**	**1.00**	**+ve**
20	**1:320 ++**	**0.89**	**+ve**
21	**1:40 +++**	**0.72**	**+ve**
22	**1:160 +++**	**0.52**	-ve
23	-ve	0.20	-ve
24	-ve	**0.37**	-ve
25	**1:20 +++**	**0.59**	-ve
26	-ve	0.25	**+ve**
27	**1:40 +++**	**0.33**	**+ve**
28	-ve	**0.65**	**+ve**
29	-ve	0.25	**+ve**
30	-ve	0.27	-ve
31	-ve	0.16	-ve

ELISA <0.25 interpreted as negative; 0.25-0.30 as suspect; >0.30 as positive.

CFT >1:10 ++ is interpreted as positive.

Bold typeface highlights positive results

The eight samples from the Portuguese abattoir (see chapter 4) were examined. All of the animals were positive on gross pathology. From these, all eight were positive by whole cell ELISA, seven by CFT and six by LAT. These sera were in poor condition, and one sample was particularly viscous, which gave a negative result by LAT and was just positive at 1:20 by CFT.

The sera samples tested as part of the EC FAIR and COST projects including the experimental infection samples were tested by a range of serological tests which are described in sections 6.5.4. to 6.5.6.

6. 5. 2. SERA FROM CBPP AFFECTED FARMS IN PORTUGAL

The 169 serum samples from six CBPP affected herds in Portugal were analysed by CFT, immunoblotting and LAT serological tests. All animals were also examined for the presence of lesions at post-mortem. Results for all six farms are summarised in Table 6. 2. and data for individual animals is given in Appendix 4.

TABLE 6. 2. SUMMARY OF SEROLOGICAL RESULTS FROM SIX CBPP AFFECTED HERDS IN PORTUGAL

Farm	No. of animals	No. of animals with CBPP lesions*	NUMBER OF SERA POSITIVE BY LAT	ELISA (whole cell)	CFT*	IBT*
1	55	30	38	50	29	47
2	25	9	10	11	9	12
3	18	9	12	17	11	17
4	19	2	2	10	6	7
5	37	35	17	34**	18	35
6	15	15	0	3	0	3

* Data kindly provided by J. Regalla. LNIV Portugal

** 1 sample not tested

Table 6. 2. shows that the proportion of cattle with lesions at post-mortem differed considerably amongst the six farms (from 10 % to 100 %). In general, serological tests detected more animals as positive for CBPP than gross pathology. However, on Farm 6 all 15 cattle had lesions, but all were negative by CFT and LAT, and only 3 were positive by ELISA and immunoblotting. All the serological tests detected approximately equal numbers of positives although their was a wide discrepancy between tests for individual animals (Appendix 4). For example there was 55 %, 88 %, 83 %, 79 %, 65 % and 100 % agreement between the CFT and LAT on Farms 1 to 6 respectively. It is worth noting that not all of the tests gave positive results for the same samples. Farm 6 was unusual in that all 15 cattle had lesions; none of them were positive by LAT or CFT, but three of the cattle were positive by both ELISA and immunoblotting. A summary table comparing total numbers of LAT positive and negative results is given in Table 6.3.

TABLE 6. 3. COMPARISON OF TESTS ON 69 SERA OBTAINED FROM SIX CBPP AFFECTED FARMS IN PORTUGAL

	CFT +VE	CFT –VE	ELISA +VE	ELISA -VE	IBT +VE	IBT -VE	GROSS PATH +VE	GROSS PATH -VE
LAT +VE	69	9	76*	2*	73	5	55	23
LAT -VE	26	65	52*	38*	48	43	45	46

*One sample not tested by ELISA

6. 5. 3. STATISTICAL ANALYSIS ON SERA FROM CBPP AFFECTED FARMS IN PORTUGAL

Correlation (kappa) coefficients showing the overall correlation between the tests conducted are given in Table 6.4. This shows that there was generally a poor correlation between the presence of lesions and any of the serological tests. Amongst the serological tests, a relatively high correlation (0.60 – 0.62) was observed between:

LAT and the CFT; the CFT and IBT; the LAT and ELISA; and the IBT and ELISA.

Thus it seems that amongst serological tests, the sensitivity and/or ability to detect antibodies present varies significantly. This might be predicted as the CFT detects mainly IgG but may also detect IgM; the ELISA detects IgG; the IBT detects IgG to selected proteins; and the LAT detects mainly IgM, but may also detect IgG, but presumably only to those fractions that react with the polysaccharide capsule.

TABLE 6. 4. COMPARISON OF SEROLOGICAL TESTS AND PRESENCE OF LESIONS BY KAPPA COEFFICIENT ANALYSIS

	CFT	IBT	ELISA	LESION
LAT	0.60	0.35	0.33	0.11*
	(0.06)	(0.07)	(0.08)	(0.08)
CFT	0.60	0.62	0.31	
	(0.07)	(0.08)	(0.08)	
IBT		0.61	0.31	
		(0.07)	(0.08)	
ELISA			0.21	
			(0.08)	

* no significant correlation (95 % confidence level).

A kappa value of 0 shows no correlation and a value of 1 shows total correlation. Values in brackets are standard errors. Where kappa values differ by more than 2 standard errors, correlation coefficients are significantly different at the 95 % confidence level.

6. 5. 4. EC COST TRIAL

One hundred and ninety two sera were tested as part of this study. These were: 56 UK negative sera, and 134 sera from Portugal that contained CFT 'false' positives in comparison with immunoblotting. It was known that eight sera were true positives and seven samples had been replaced with dilutions of serum from a goat that had been inoculated with *M. mycoides* subsp. *mycoides* SC.

The tests compared were the LAT, CFT, modified CFT and competitive ELISA and immunoblotting. Each test was carried out either by the OIE recognised laboratory, or where the specialist tests had been developed; the CFT and immunoblotting was carried out at LNIV, Portugal; the cELISA by CIRAD, France; the modified CFT by IZS, Teramo, Italy. The modified CFT, developed at IZS, Teramo used reduced concentrations of haemolysin (2 units), complement (2 units) and red blood cells (2 %) compared to that described by Campbell and Turner (1953) of 12 units, 2.5 units and 6 % respectively.

The 56 UK negative serum samples were negative in all tests. The eight positive control samples were all positive using the CFT; but only six were positive by LAT and the modified CFT; however only three were positive by the cELISA. The neat positive goat serum was detected by the LAT, immunoblotting (weak positive), the modified CFT at 1/40 and with 74 % inhibition in the cELISA. The cELISA also gave a positive result with goat sera diluted 1 in 4. The results from the Portuguese sera were more complex and are given in Table 6.5.

The serum samples originally found to be positive by CFT but negative by immunoblotting contained 59 samples that were negative by all serological tests. Interestingly, one sample, number 103, was positive by all the tests (other than immunoblotting) used in this trial. Amongst the remaining serum samples 3 were positive by cELISA; 31 by the modified CFT, 52 by the traditional CFT and 13 by LAT. Of the 13 LAT positives 9 were negative in all other serological tests and four were positive in one or both of the CFT tests. All 'false' positives were negative by cELISA. Thus, it appears that, as was concluded in the section 6.5.3. above, the serological tests compared vary in their sensitivity and/or specificity for detecting antibodies to *M. mycoides* subsp. *mycoides* SC. Analyses between all these

techniques showed that there was no significant correlation: a serum with a low value in one test can give a high value in another.

TABLE 6. 5. COMPARISON OF RESULTS OBTAINED USING DIFFERENT SEROLOGICAL TESTS IN THE EC COST TRIAL

Sample Number	LAT Result	CFT[1] Log_{10}	Modified CFT[2] Log_{10}	cELISA[3] (%)
1	-ve	**2.05**	**2.05**	21.72
4	-ve	**1.45**	1.08	32.52
7	-ve	**1.38**	1.08	25.36
8	-ve	**1.38**	1.15	40.44
10	-ve	**1.38**	0.	31.08
11	**+ve**	1.08	0	31.43
12	-ve	**1.75**	**1.90**	24.84
15	-ve	**1.38**	1.15	40.50
16	-ve	**1.38**	1.15	22.70
17	**+ve**	1.08	0	26.11
19	-ve	1.08	**1.38**	**67.76**
20	-ve	**2.28**	**2.65**	36.11
21	-ve	**1.38**	0	28.65
23	**+ve**	0	0	13.92
24	**+ve**	0	**1.38**	33.51
26	**+ve**	0	0	37.78
27	-ve	**1.98**	**1.60**	29.46
28	-ve	**1.38**	0	23.63
32	-ve	**1.38**	0	**55.63**
33	-ve	**1.98**	0	43.79
34	-ve	**1.75**	**1.38**	23.17
35	-ve	**1.38**	**1.30**	27.21
36	**+ve**	**1.75**	0	24.09
37	**+ve**	**1.45**	0	43.67
38	-ve	**1.45**	**1.30**	23.22
41*	-ve	0	0	**60.41**
42	-ve	1.15	**1.45**	22.87
45	-ve	**1.68**	**1.68**	16.45
46	-ve	**1.68**	**1.30**	11.68
48	-ve	**1.45**	0	26.47
49	-ve	**1.38**	1.15	29.35
50	-ve	**1.38**	**1.38**	30.45
51	-ve	**1.68**	1.08	37.38
52	-ve	**1.68**	**1.45**	30.62
55	-ve	**1.38**	**1.38**	22.28
56	-ve	**1.83**	**1.90**	25.97
57	-ve	**1.98**	**2.05**	28.74
58	-ve	**1.38**	0	13.90
59	-ve	**2.05**	**2,05**	32.95
60	**+ve**	**1.68**	1.08	24.64
63	-ve	**2.05**	1.30	28.52
64	-ve	**1.68**	**1.75**	30.95
66	**+ve**	1.08	0	12.29
68	-ve	**1.45**	**1.68**	34.05
69	-ve	**1.98**	**1.90**	34.44
71	-ve	1.15	**1.60**	25.36

TABLE 6. 5. COMPARISON OF RESULTS OBTAINED USING DIFFERENT SEROLOGICAL TESTS. (EC COST TRIAL CONTINUED)

Sample	LAT Result	CFT[1]	Modified CFT[2]	cELISA[3]
		Log_{10}	Log_{10}	%
72	-ve	**1.45**	**1.60**	28.52
76	-ve	**1.38**	**1.38**	40.59
77	**+ve**	1.08	0	13.23
79	-ve	**1.45**	**1.38**	33.11
83	-ve	**1.38**	1.08	34.70
87	-ve	**1.38**	0	24.57
90	-ve	**1.68**	**1.90**	29.23
91	-ve	**1.75**	**1.45**	35.21
97	-ve	**1.38**	0	18.59
98	-ve	1.15	**1.45**	26.16
100	-ve	**1.38**	0	31.23
103	**+ve**	**1.45**	**1.45**	**54.57**
108	-ve	**1.45**	**1.45**	26.22
109	-ve	**1.68**	**1.98**	18.25
112	-ve	**1.38**	0	24.61
113	**+ve**	1.15	0	31.54
115	**+ve**	1.08	0	34.75
116	-ve	**1.45**	0	31.02
117	**+ve**	0	0	35.95
120	-ve	**1.38**	0	29.71
124	-ve	**1.68**	0	22.95
**127 & 131	**+ve/+ve**	**2.58/2.58**	**2.65/2.65**	46.88/47.91
**128 & 132	-ve/-ve	**1.98/1.98**	**1.98/1.75**	48.48/**51.63**
**129 & 133	**+ve/+ve**	**2.58/2.58**	**2.58/2.50**	**59.36/60.90**
**130 & 134	**+ve/+ve**	**1.38/1.38**	1.15/1.15	35.43/37.15

Sera from the 56 UK cattle and 59 Portuguese cattle that were negative by all tests are omitted from this table.

*Goat sera from animal inoculated with *M. mycoides* subsp. *mycoides* SC antigen diluted 1 in 4.

** Duplicated control positive *M. mycoides* subsp. *mycoides* SC samples.

[1]CFT data kindly provided by J. Regalla, LNIV, Portugal; data were originally scored as 0 to ++++ at each serum dilution. They were converted to log_{10} values by F. Thiaucourt, CIRAD, France; based on the reciprocal of the highest titre giving any positive result and the degree of precipitation. A reaction of + at a 1/20 dilution was converted to a log_{10} value of 1.3 and was the lowest value for a positive result.

[2]The modified CFT (Tittarelli *et al.*, 1999) was carried out by P. DeSantis, IZS, Teramo, Italy and converted to log_{10} values by F. Thiaucourt, CIRAD, France as described above.

[3]The cELISA was carried out by F. Thiaucourt, CIRAD, France. A positive result was regarded as >50 %; 45-50 % was considered as inconclusive.

6. 5. 5. SERA FROM PNEUMONIC CATTLE IN HUNGARY – UNKNOWN STATUS

All the sera tested were negative by LAT, whole cell ELISA, tween 20 treated ELISA and CFT for CBPP, as described in section 6.3.

6. 5. 6. EC FAIR PROJECT EXPERIMENTAL INFECTION

Cows 1 and 4

The LAT, whole cell and tween 20 ELISA remained negative throughout the experiment for both animals. Other information relevant to the experimental infection in these cattle was provided by IZS, Italy. The CFT also remained negative throughout the experiment, as was culture from the nasal swabs. PCR gave some positive results when used directly on the nasal swabs, following enrichment, and directly from the blood on 7 and 8 occasions respectively. No clinical symptoms or disease were observed during the experiment.

At post-mortem, in cow 1, some minor adhesions were observed on the chest wall, but the lungs appeared normal. In cow 4, pneumonia was observed with a thickening of interlobular septa and a lesion was recorded. No characteristic CBPP lesions were observed. Culture, PCR and ICC were negative for both cows.

Cow 2

The LAT became positive 1 week post intubation and remained positive throughout the experiment. The tween 20 ELISA became positive by week 3 and declined by week 10, with a slight increase for 2 weeks prior to slaughter. The whole cell ELISA was positive by week 1, peaking at weeks 2 and 3 before declining by week 5; again a slight increase was observed for 2 weeks prior to slaughter (see Figure 6.1.).

The CFT also became positive at week 1, peaked at week 3 and started declining by week 4 but remained positive throughout. Culture remained negative throughout. PCR detected positives from nasal swabs on weeks 3 and 5 and from blood on weeks 4, 5, 6, 8, 10, 19, 20 and 21. At post-mortem some congestion of the upper respiratory tract was observed as were minor adhesions and a general

thickening of pleura at the lobe level. No characteristic CBPP lesions were observed. Bacteriology was negative but PCR and ICC were positive on lung tissues. No clinical symptoms or disease were observed during the experiment.

FIGURE 6. 1. COMPARISON OF ELISA METHODS AND LAT USING SERA FROM EXPERIMENTAL INFECTION COW 2

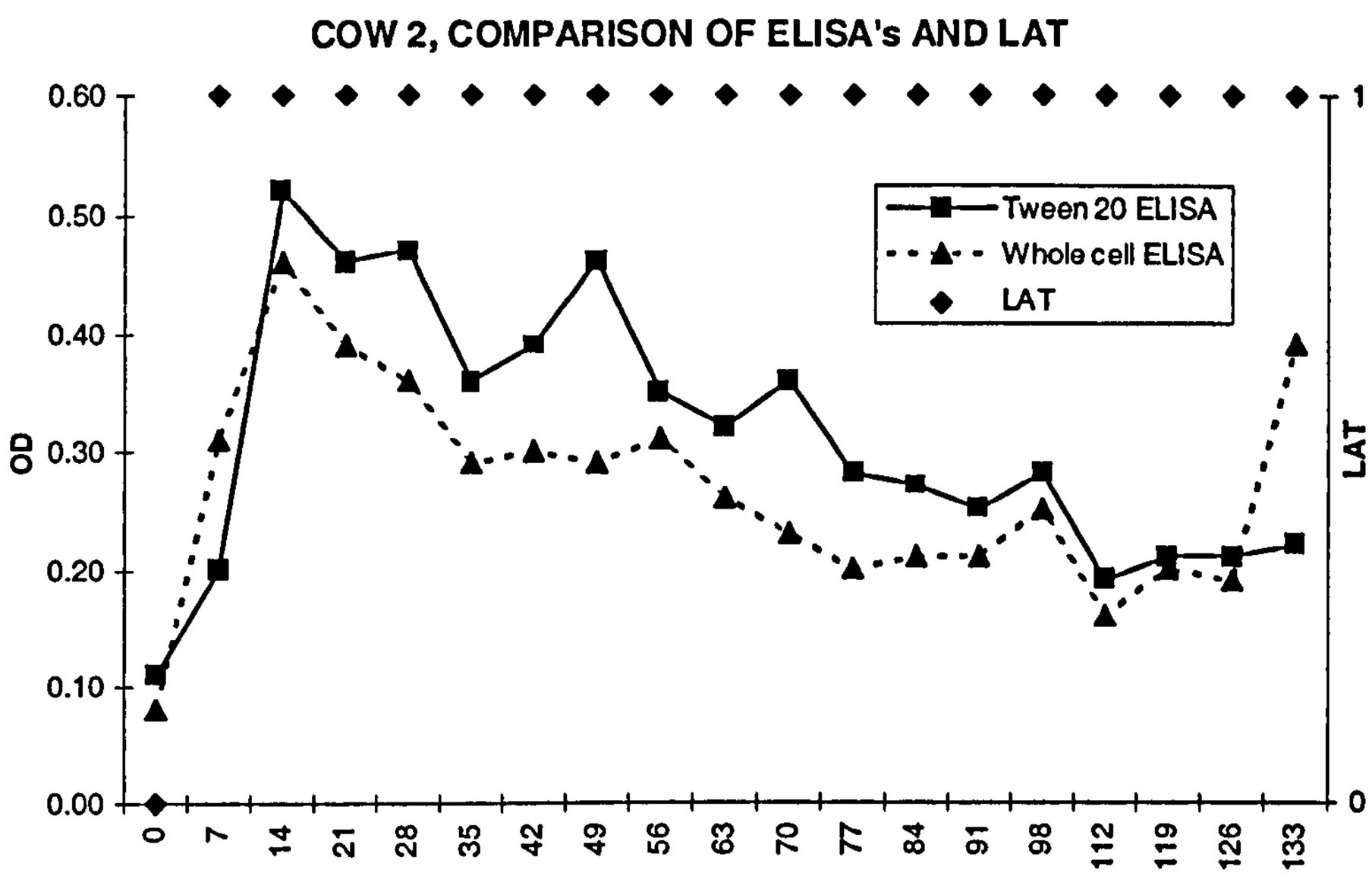


Figure 6.1 shows the comparison of ELISA's and the LAT for sera obtained from cow 2 during the experimental infection.

The ELISA cut-off points may be taken as an OD of 0.3, although the exact value has still to be determined.

For the LAT 0 is negative, 1 is positive.

Cow 3

The LAT became positive 2 weeks post intubation and remained positive throughout the experiment. The tween 20 ELISA remained negative, but showed an increased OD over the experimental period. The whole cell ELISA was positive at week 1, but declined and remained negative; a slight increase was observed for 2 weeks prior to slaughter (see Figure 6.2.).

The CFT became positive at week 3, peaked at week 4 and became negative by week 10. No clinical symptoms or disease were observed during the experiment. Culture remained negative throughout. PCR detected positives from nasal swabs on week 2 only and from blood on weeks 3, 5, 6, 7, 10, 19, 20 and 21. At post-mortem a small volume of clear exudate was seen. Filaments of organised fibrin of the right apical lobe were recorded, as were some adherences and a general thickening of pleura at the lobe level. No characteristic CBPP lesions were observed. Bacteriology, PCR and ICC were negative.

FIGURE 6. 2. COMPARISON OF ELISA METHODS AND LAT USING SERA FROM EXPERIMENTAL INFECTION COW 3

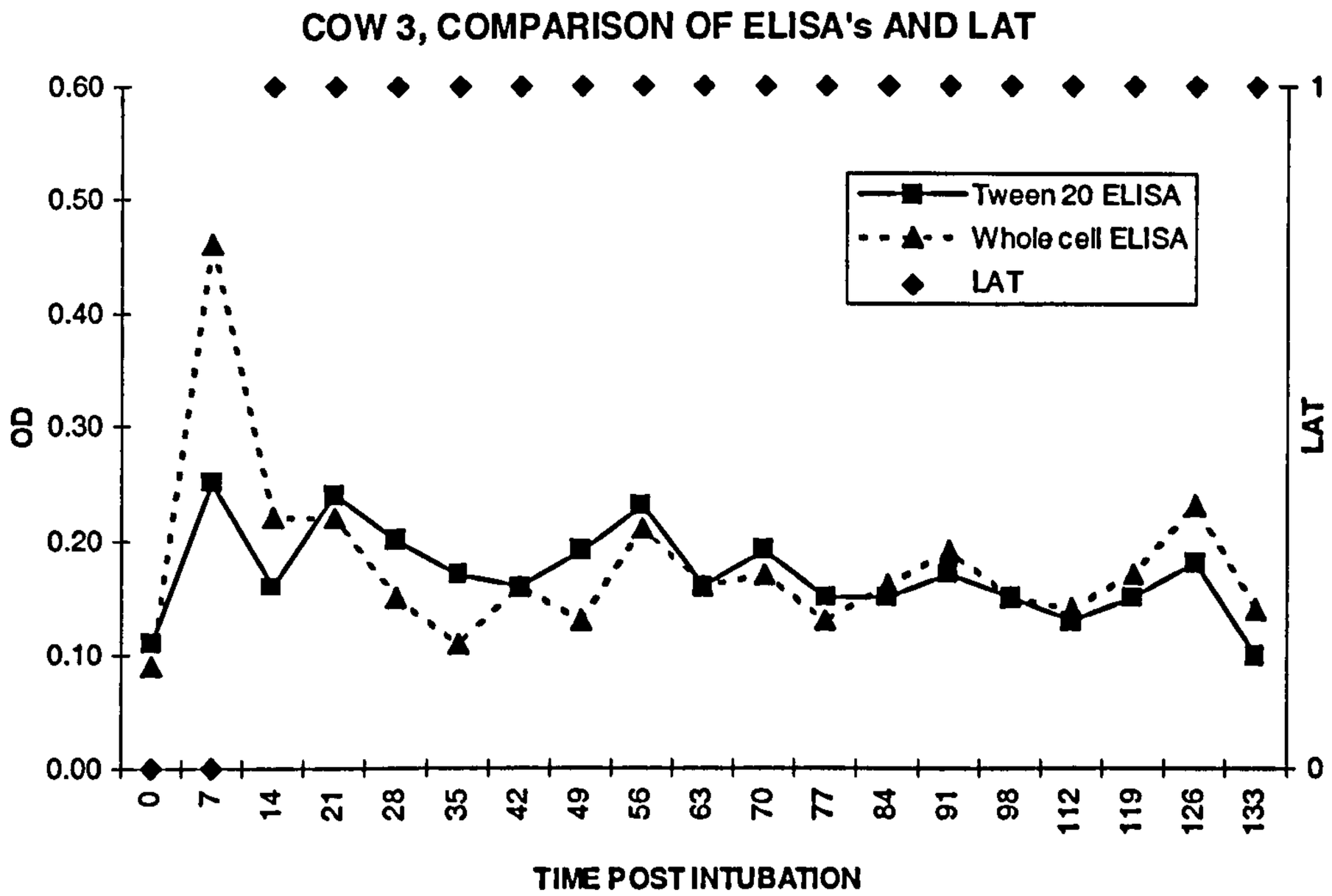


Figure 6.2 shows the comparison of ELISA's and the LAT for sera obtained from cow 3 during the experimental infection.

The ELISA cut-off points may be taken as an OD of 0.3, although the exact value has still to be determined.

For the LAT 0 is negative, 1 is positive.

6. 6. DISCUSSION

In the development of diagnostic tests, it is useful to have an idea of the test qualities required. Ideally tests should be highly specific, sensitive and simple to perform and interpret. In practice, for serological tests, highly specific and highly sensitive tests are difficult to achieve as an increase in sensitivity usually leads to increased numbers of false positive results and hence a decreased specificity.

There is a place for a number of serological tests for detecting CBPP and for its control. In countries such as the UK, where CBPP is not present, the more sensitive screening test, currently the CFT, can be used either to confirm the disease free status, or for use in import tests. Where distance and communications is not a problem, these tests can be performed at central laboratories, which have the required expertise and the facilities for automation, and therefore the ability to process a large number of tests. Where positive results are obtained a more specific test such as immunoblotting can be used to confirm the result. However, in countries such as Africa, where CBPP is endemic and an increasing problem, this system is not effective for many reasons, including distances involved, lack of skilled staff and adequate testing facilities. The CFT is performed in a number of laboratories, but until recently no other tests were used. Thus there was no means of confirming CFT positive results. The use of an ELISA system such as the cELISA (Le Goff and Thiaucourt, 1998) in Africa offers an alternative test, which may also be used as a confirmatory test. However, one of the main difficulties in Africa is the distances involved between the animals and diagnostic laboratories, and the inability to retrace positive animals, as nomadism and trekking is part of the African culture. A pen-side test such as the LAT would be extremely useful in this situation, where rapid diagnosis can be made.

In this thesis a number of CBPP serological tests have been used on a variety of serum sources, providing comparative data on old and new CBPP serological tests. The CFT, which is currently the OIE recommended test, produced more positive results overall than any other serological test. However, on the samples from the farms in Portugal, many samples were CFT negative but positive by other tests and by gross pathology. For example on farm one (Appendix 4), where 55 animals were tested, 5 samples were CFT negative, but positive for

lesions, and by LAT, ELISA and immunoblotting. Twenty-one additional CFT negative tests on farm one were positive by at least two other tests. This suggests that the CFT may only be about 53 % sensitive in comparison with the 70 % previously reported (Stärk *et al.*, 1994). On Farm 5, (where CBPP lesions were reported in all but two of the animals from that farm), the figure falls to 45 % sensitivity compared with other tests. In contrast, the CFT tested in the EC COST trial gave false positive results on 52 of the 134 (39 %) samples tested. Although the CFT is the OIE recommended test, this work shows some of the difficulties that arise in diagnosing the disease, when you have a test that is neither specific or sensitive. In addition the CFT has many disadvantages in the standardising and performing of the test and can only be performed in a specialised laboratory.

The modified CFT (Tittarelli *et al.*, 1999) showed fewer false positive reactions than the conventional CFT in the EC COST trial, but also missed two of the positive control samples. The advantage of this test over the conventional CFT is the reduction in the amount of reagents required.

The cELISA (Le Goff and Thiaucourt, 1998) has recently been accepted by the OIE. The test, supplied by CIRAD, is however complicated, being supplied as raw reagents. Reading of the tests also requires specialist equipment, interpretative and data manipulation skills. In the EC COST trial the cELISA detected only 3 false positive results, although one of those (sample 103) was positive by all other tests except immunoblotting. From this information the test looks highly specific, but it totally missed two of the positive controls and three positive controls were below the cut-off point in the inconclusive range. Reports by Yaya *et al.* (2000) indicate that the cELISA has similar sensitivity to that of the CFT; however they also reported that in experimental infections the cELISA failed to detect some cattle that developed typical CBPP lesions, although an increase in titre was observed.

The whole cell ELISA (see sections 5.2.7) was used for the sera tested from Uganda and from the 6 Portuguese farms. Sixteen of the 31 samples were positive by ELISA and 18 by CFT. Statistical analysis (Table 6.4) showed kappa coefficients of 0.61 and 0.62 between the ELISA, immunoblotting and CFT, which compares favourably with the other tests. However, the ELISA was not the most

sensitive of tests when the experimental infection was monitored, although it did detect immune responses in two affected cattle. Currently, this ELISA is only a research tool, as it is known to have some cross-reactions with *M. bovis.* However, ELISA's in general could be modified for field use, with the colour development and interpretation being read by eye and matched to colour cards. The use of sample titration could also help determine a titre.

The tween 20 ELISA (see sections 6.3), was a development of the whole cell ELISA, and was used as the removed tween soluble antigens were the main cause of cross-reactions with *M. bovis.* It would appear that the sensitivity of both the whole cell ELISA and tween 20 ELISA could be increased, although the positive control for each ELISA gave an OD of 1.

The LAT (chapter 5) has given some interesting results and shows potential as a screening test for CBPP. On the Portuguese farms the kappa coefficient was 0.60 compared to the CFT, with lower values for the immunoblot, ELISA and gross pathology. However, in the EC COST trial the LAT gave only only 13 (9.7 %) false positives compared to the 39 % of false positives in the CFT. It did, however, miss two of the eight positive controls; the cELISA missed five. In the experimental infection, the LAT detected antibody in two cattle within the first two weeks. The LAT remained positive throughout the experiment, showing the ability of the LAT to detect early infection and still record positive results, when other serological test titres had declined.

There was an 81 % agreement between the CFT and the LAT and a 77 % agreement between the ELISA and the LAT on sera from Ugandan cattle. This demonstrated that the LAT using an extract obtained from a European strain would also detect immune responses from Ugandan cattle affected with African strains, which have been shown to be different to European strains (Frey *et al.*, 1996; Houshayami *et al.*, 1997).

The few sera tested from Italy showed a good correlation between serological tests.

Only two false positive LAT results were detected in the Hungarian and UK sera tested. One of those sera was of dubious quality. This would indicate that the test is very specific.

The LAT is cheap, rapid and can be performed by relatively unskilled personnel and can be used in a simple mobile laboratory close to the cattle, thus giving it several practical advantages. As a rapid screening test, it shows considerable potential. Whole blood was used (data not shown) on some samples from the experimental CBPP infection giving identical results to those obtained using serum, although the agglutination was more difficult to see. This could be addressed by using different colour beads and waxed card. This would then make the test a true pen-side test.

Evaluating test effectiveness presents several difficulties, including insufficient material of known status, inconclusive existing tests and the lack of a "gold standard" test. In this thesis a wide range of sera have been tested from different well-documented sources. These included the naturally occurring CBPP infections in Portugal, and Italy where details of lesions and other tests were known. Time studies were performed on the experimental infection. Samples from CBPP affected herds in Uganda demonstrated the LAT would work with antibodies to African strains. Sources of CBPP negative sera were also important for evaluating serological tests and bovine samples from the UK and Hungary provided plenty of samples where the rate of false positivity in true negative populations could be determined.

6. 7. CONCLUSION AND FURTHER WORK

No single serological test detected all CBPP affected cattle. Whole cell ELISA and immunoblotting were more sensitive than the CFT and LAT in the trial carried out using sera from Portuguese farms. However, LAT gave more positive tests than the CFT on the sera from Portuguese farms, in contrast to the overall to the results obtained from the EC trial, where CFT produced the most positive results. Thus, as a rapid test for use in field laboratories the LAT appears to offer considerable potential. This test would also appear suitable for use with whole blood and might therefore be of great value as a 'pen-side' test.

Field evaluation of the test is now required. In relation to tests in African cattle, it will be important to determine the reaction of vaccinated and or antimicrobial treated cattle. One would expect the vaccinated animals to give a

positive LAT reaction. Whilst it would be useful to be able to differentiate between vaccinated and CBPP affected cattle, the test could also be used to determine if the vaccination had successfully produced an immune response that may offer protection against CBPP. It is unlikely that antimicrobial treatment would affect the outcome of the LAT. For field use, it may be possible to further increase the convenience of the LAT by drying the antigen-coated latex beads onto test cards. Test blood samples may then be simply applied to the cards.

CHAPTER 7

7. 1. GENERAL DISCUSSION AND FURTHER WORK

Many mycoplasmas are important pathogens of economic significance in domestic animals. This thesis has been concerned with aspects of the diagnosis and control of CBPP and *M. bovis* infections. These are currently the most important of the mycoplasma infections of cattle (see sections 1.4 and 1.5). However strategies for their control differ widely. In Africa, the containment and occasional slaughter of affected cattle and the use of vaccines are the methods used in attempts to control CBPP. In contrast, a slaughter and compensation policy was used to eradicate CBPP from Italy and is in use in Portugal, and would be used in the UK should cases arise. For a slaughter and compensation scheme to be effective, efficient and early diagnosis of the disease is paramount. *M. bovis* is endemic worldwide and currently no effective control mechanisms are in place. Antimicrobials are widely used in treating infections, but they are generally not effective, although they may temporarily relieve the symptoms and give the animal time to develop its own immune defences.

The contribution of the work described in this thesis to the diagnosis and control of *M. mycoides* subsp. *mycoides* SC and *M. bovis* infections are:

(i) the evaluation of a PCR giving improved speed and reliability of *M. bovis* identification.

(ii) the development of an efficient antimicrobial sensitivity assay for mycoplasma and its application to the determination of sensitivity amongst a diverse range of *M. bovis* and *M. mycoides* subsp. *mycoides* SC isolates. In this study the effectiveness of antimicrobials currently used to treat *M. bovis* infections in the UK was determined (*in-vitro*). The study also identified potential candidate antimicrobials for treatment of CBPP in Africa and Europe (if such treatment became an option) and showed the likely speed with which increased mycoplasma antimicrobial resistance would be acquired.

(iii) a critical evaluation of a range of CBPP diagnostic methods: PCR; CFT; modified CFT; cELISA; whole cell ELISA; tween 20 ELISA; immunoblotting; and LAT.

(iv) the development of a simple LAT for CBPP based on a culture supernatant fraction, which was identified as the capsular polysaccharide antigen.

7. 2. CONTROL OF *M. BOVIS AND M. MYCOIDES* SUBSP. *MYCOIDES* SC INFECTION

It was just over 100 years ago that Nocard and Roux (1898) succeeded in culturing the causative organism of CBPP when the disease was having a severe effect on the cattle industry of many European countries. It has since been eradicated from most European countries, with a possible pocket of infection remaining in Northern Portugal. There have been no outbreaks of CBPP in Britain for more than 100 years but it is clear from recent outbreaks in Europe that the disease still presents a real threat to the British cattle industry. Although there was only one reported case of CBPP in Europe during 1999 and none in 2000, there have been reports in Europe during every decade of the last century (Nicholas *et al.*, 2000a). With the formation of the European Union, and increased European trading with fewer restrictions means the possible threat of importing the disease into Britain is increased. CBPP would undoubtedly have a devastating effect on the British cattle industry.

In this study, the disease was observed in Portugal (chapter 4), thus providing an insight into what may happen if cases occurred in the UK. The way the disease can spread relatively easily by aerosol transmission downwind to neighbouring farms, the posture of the animals and the dry cough have all been witnessed. Aerosol transmission of CBPP infection is a major risk factor, although in the UK, farms are not usually in such close proximity as those observed in Bougado in Portugal and therefore the risk may be less. However, in the UK, the clinical symptoms, the cough and posture would initially be mistaken for another pneumonic disease and would be treated with antimicrobials, possibly masking the disease and facilitating its longevity and spread. At the post mortems in Portugal, the previously described gross pathology was recorded including unilateral lung

infections, presence of sequestra and the marbled appearance of the lung. In the UK, routine checks by meat inspectors at the abattoir may be where CBPP is first detected. Meat inspectors and veterinarians are aware of CBPP but few have witnessed the gross pathology first-hand. Photographs of the pathology observed in Portugal are now shown to new veterinarians at induction courses run by VLA, and have also been supplied to some regional laboratories. Material was also collected from Portugal so that traditional diagnostic methods, culture, PCR and CFT could be carried out. This also provided some material to evaluate new diagnostic methods such as ICC, ELISA and the LAT. These tools are invaluable in diagnosing CBPP. When suspect cases are reported in the UK, culture and PCR are used first, on lung material, pleural fluid and nasal swabs and any other affected tissue. The PCR gives a rapid 'preliminary' result, but requires culture confirmation. In the mean time serology, CFT and new ELISA tests are performed on sera from the affected animal and any contact animals. However, as reported in chapter 6, none of these tests are 100 % successful, but with a combination of the old and new tests it is likely that an affected herd, if not individual animals would be detected. If CBPP entered the UK, there are several scenarios of its course:

- it is possible that it remain undetected for some time and only be detected at post-mortem, thus facilitating its spread by cattle movement and sales;
- it is also possible that naïve cattle will be seriously affected and the disease diagnosed rapidly and the cattle slaughtered before the disease spreads;
- it is probably more likely that as in Portugal, the farmer will notice the failure of the animals to do well, with lower milk yields and that CBPP will only be considered after other diseases have been discounted and the animals have failed to respond to antimicrobial treatment.

Once CBPP has been diagnosed in the UK, the slaughter policy and tracing of animal movements would be quickly implemented, thus quickly eradicating the disease. This would be carried out more rapidly than was observed in Portugal where negotiations about slaughtering and compensation appeared to be pro-longed over many months, before animals were slaughtered.

An experimental infection with CBPP (section 6.2.1) produced two cattle with a detectable immune response and *M. mycoides* subsp. *mycoides* SC was culturable on occasions, however no 'gross pathology' was present. This experimental infection produced a sub-clinical infection and provided characterised material for evaluation (chapter 6) of the LAT developed in chapter 5. It is this level of infection that presents a real threat for importation of disease.

The CBPP experimental infection carried out in Italy under the EC FAIR project CT95-0711 followed the infection procedure of Abdo *et al.* (1998) with direct intubation of culture into the lung. This method is probably much less effective than aerosol methods (Hudson, 1971), in which calves breathe in the challenge organisms. Even so, little information has yet been obtained on incubation times or how long it takes for the typical gross pathology to develop. It has been reported (Egwu *et al.*, 1996) that incubation times may range from 5-207 days although an incubation time of 207 days represents the extreme case. This information is based on a shipment of cattle from England to Australia more than 100 years ago, when an animal developed the disease after arrival in Australia (Egwu *et al.*, 1996).

Much discussion has recently taken place about the differences between European and African *M. mycoides* subsp. *mycoides* SC isolates. Undoubtedly, differences exist in aspects such as Insertion Sequence (IS) elements (Frey *et al.*, 1996) and ability to oxidise glycerol (Houshaymi *et al.*, 1997) and possibly virulence (Provost *et al.*, 1987). Even though cattle mortality is rare in affected European countries compared with African countries, it is evident that the European *M. mycoides* subsp. *mycoides* SC still causes typical CBPP disease with affected cattle showing similar gross pathology (Nicholas *et al.*, 1996). Other factors such as breed of cattle, husbandry, stress and possibly previous antimicrobial treatment must affect the pathogenesis of CBPP.

Mycoplasma bovis was recognised as a cause of disease in cattle relatively recently, in 1961. Although like *M. mycoides* subsp. *mycoides* SC it is associated with pneumonia in cattle, there are substantial differences between the organisms in relation to the nature of the disease caused and diagnostic methods for detection. *M. bovis* has been reported to be present almost world-wide and is not

only a major cause of pneumonia, but also of arthritis and keratoconjunctivitis in calves, and mastitis and reproductive disorders in cattle (Nicholas *et al.*, 2000b). It was diagnosed in approximately 25 % of pneumonic calves in Britain using an indirect ELISA (Nicholas *et al.*, 2000b). With a few notable exceptions, including *M. mycoides* subsp. *mycoides* SC, it has generally been assumed that mycoplasmas play a secondary role in infection, particularly in complex pneumonic infections where other bacteria and viruses are identified. However, there is increasing evidence that mycoplasmas, such as *M. bovis* may be primary pathogens. *M. bovis* causes infection experimentally (Pfutzner and Sachse, 1996; Stipkovits *et al.*, 2000b), and in farm studies *M. bovis* was associated with persistent infections in which other pathogens were absent (Nicholas *et al.*, 2000b).

Currently, diagnosis is usually based on serum ELISA tests, and a commercial ELISA test (Vetoquinol) is available. However, the organism grows well in culture and may be isolated from lungs, or joints, but is only shed and detected intermittently from nasal cavity swabs (Ter Laak *et al.*, 1992). In contrast to *M. mycoides* subsp. *mycoides* SC, it does not ferment glucose and also produces a film. Generally, confirmation of identification is by GIT or IFAT using specific polyclonal rabbit sera (see section 2.1.2.). However, the presence of variable surface proteins (*vsps*) (Behrens *et al.*, 1994) has on a few occasions complicated diagnosis (see Section 2.1.2.). In addition, *M. bovis* and *M. agalactiae* are very closely related, both biochemically and genetically, with only 8 bases different in the 16S rRNA gene (Johansson *et al.*, 1996b). An incorrect diagnosis would have political implications, as *M. agalactiae* is exotic to Britain. Although *M. bovis* is usually isolated from cattle and *M. agalactiae* from sheep and goats, several reports of *M. bovis* in goats have been received from Europe (Petridou *et al.*, 1998). To overcome potential problems in the identification of isolates a PCR was developed (chapter 2) to detect and differentiate *M. bovis* and *M. agalactiae.*

7. 3. PCR METHODS FOR MYCOPLASMA IDENTIFICATION AND DETECTION

Since the first PCRs were described, many have been developed for a wide range of mycoplasmas, including members of the *M. mycoides* cluster and some specific groups within the cluster (Bashiruddin *et al.*, 1994a; Dedieu *et al.*,

1994; Hoetzel *et al.*, 1994; Persson *et al.*, 1999). The most widely used *M. mycoides* subsp. *mycoides* SC PCR is based on the CAP21 gene fragment (Bashiruddin *et al.*, 1994a). At the beginning of the work described in this thesis, a PCR method for *M. bovis* had recently been described (Chavez González *et al.*, 1995; Johansson *et al.*, 1996a). The PCR was optimised and its specificity determined using isolates of various *Mycoplasma species.* Different hot-start PCR methods were tried to improve the specificity of the PCR and it was determined that heating at 94°C for 4 minutes before the addition of the *Taq* enzyme was required for the PCR method to be specific.

A further aspect considered in the development of the PCR methodology was the mycoplasma DNA extraction procedure. Standard DNA extraction procedures (Bashiruddin, 1998) require time consuming incubation, separation and precipitation stages, with some protocols taking 48 hours, thereby delaying mycoplasma species identification in the laboratory. PCR tests directly on cells in medium showed that some inhibitory effect was present, preventing successful PCR amplification. Presumably the growing cells secreted some inhibitors into the medium that affected the PCR. It was also shown that extracted DNA in 'fresh' Eaton's medium and cells washed once in PBS resulted in successful amplification. The PCR developed in chapter 2 was introduced for routine use at VLA in 1997, and has been used to confirm the identity of approximately 400 *M. bovis* isolates. Use of the *M. bovis* PCR has proved useful in providing an initial identification that was later confirmed by GIT, or in confirming GIT results. On a few occasions the PCR has produced amplicons from mixed cultures, which by further culturing and cloning were shown to contain *M. bovis.*

Subsequent to the development of the PCR based on the 16S rRNA gene and described in this thesis, Subramaniam *et al.* (1998) published two PCR methods, one for *M. bovis* and the other for *M. agalactiae.* These PCRs were based on the *uvr*C gene, which showed more variation in sequence between the two organisms than the 16S rRNA gene. An inter-laboratory trial of the 16S rRNA and *uvr*C based PCRs was conducted as part of EC COST Action 826 on ruminant mycoplasmoses. DNA from *M. bovis* and *M. agalactiae* strains isolated in different countries was sent to participating laboratories. Results showed some inter-laboratory variation,

where the identification of some strains was not conclusive, but both methods were effective.

The value of the PCR methods in the UK was shown in November 1999. A mycoplasma was isolated from milk taken from a goat with mild mastitis in Kent. The isolate did not ferment glucose or hydrolyse arginine and produced a film, suggesting *M. agalactiae.* However, both the *M. bovis* PCR procedure developed in this thesis and the PCR method of Subramaniam *et al.* (1998) showed that the isolate was in fact *M. bovis.* Further sampling at the farm investigations failed to recover similar isolates.

7. 4. ANTIMICROBIAL SENSITIVITY TESTING

Antimicrobials, many of which target the bacterial cell wall, are frequently used to treat and control diseases in humans and animals. However, as mycoplasmas lack a cell wall many antimicrobials are not effective. One problem with antimicrobial use for disease control is that it may lead to the development of clinically healthy carrier animals spreading the diseases and also to the development of antimicrobial resistance. New more effective antimicrobials may be beneficial and FAO Emergency Prevention System (EMPRES) recommended research into the role of chemotherapy for CBPP (Rweyemamu and Benkirane, 1996). In chapter 3 of this thesis, the *in-vitro* effectiveness of five antimicrobials was tested against *M. mycoides* subsp. *mycoides* SC and *M. bovis* isolates. The MIC and MMC levels were determined using an improved microtitre method. This method meets all the recommendations made by the IRPCM chemotherapy committee for antimicrobial testing (Hannan, 2000).

Results of antimicrobial testing (chapter 3) showed distinct differences between *M. mycoides* subsp. *mycoides* SC and *M. bovis* isolates. The *M. bovis* isolates, all from Britain, showed that antimicrobial resistance had developed to tilmicosin, oxytetracycline, spectinomycin and to a lesser extent florfenicol. In contrast, little resistance had been developed by *M. mycoides* subsp. *mycoides* SC, although some isolates were resistant to spectinomycin. Repeated sub-culturing of *M. mycoides* subsp. *mycoides* SC at the MIC level quickly induced substantial resistance to spectinomycin, but not to the other antimicrobials and no cross-resistance was observed. Although many differences exist between *M. mycoides*

subsp. *mycoides* SC and *M. bovis*, it may be argued that the development of resistance in the two groups is likely to be similar. If this is the case antimicrobial resistance in *M. mycoides* subsp. *mycoides* SC is likely to develop quickly, if the use of antimicrobials in Africa becomes widespread. The newer fluoroquinolones such as danofloxacin may be useful in strategic and possibly prophylactic treatment; however preliminary studies on a farm continually affected by *M. bovis* showed that the use of danofloxacin had little benefit, although ongoing viral diseases may have complicated the clinical picture (Nicholas *et al.*, unpublished results).

7. 5. DIAGNOSTIC METHODS FOR CBPP

For the confirmation of important diseases such as CBPP it is still necessary to demonstrate the presence of the organism to the regulatory authorities in order for control measures to be initiated. However, culturing and identification of an organism is not always successful. This may be due to the phase of infection, the presence of sufficient organisms at the relevant site, the culturing method or presence of other 'contaminating' organisms that may outgrow or inhibit the *in vitro* growth of the specific disease-causing organism. Immunological diagnosis of a disease is often preferable although not conclusive. Animals mount a specific immune response against most infectious agents and it is the detection of this specific immune response that indicates that animals have been exposed to that specific antigen. However, the type and length of immune response varies from animal to animal, antigen to antigen and on antigen exposure levels.

The whole cell indirect ELISA for *M. bovis* (Nicholas *et al.*, 2000b) has been in use at VLA (Weybridge) for many years and appears to have been very effective. It has often led to the diagnosis of *M. bovis* pneumonia before *M. bovis* isolation. In addition it has been used to detect an immune response in milk, even identifying affected mastitic quarters (Byrne *et al.*, 2000). The success of this ELISA may possibly be attributed to the lack of cross-reacting antigens with other pneumonia and mastitic causing organisms.

The limitations of the current CBPP immunodiagnostic tests are known, (see section 1.5.7.1.). The CFT is the OIE recognised test, but has serious disadvantages in sensitivity and ease of performance. The competitive ELISA developed by Le Goff and Thiaucourt (1998) has been considered for use as an OIE

recommended test but while easier to perform has similar sensitivity. The immunoblotting test (Regalla *et al.*, 1996b) appears to be the most sensitive and specific laboratory test, but cannot easily be scaled up for mass screening.

One aim of this thesis was to develop a simple field/penside test to rapidly identify affected herds and possibly individual animals so that immediate action can be taken to control the spread of CBPP. In chapter 5 a specific antigen from *M. mycoides* subsp. *mycoides* SC was identified. This was then used to develop a simple immunodiagnostic test, the LAT test that could be carried out rapidly and in the field by 'unqualified' people. The simplicity and low cost of such a test may increase the level of screening for CBPP in affected countries, enabling quicker control action to prevent further spread of the disease. It is accepted that such a test is unlikely to be more specific and sensitive than laboratory based tests; however when evaluated in chapter 6, the test performed particularly well in comparison with the CFT, but also with the ELISA, cELISA and immunoblotting particularly in the EC trial in section 6.5.4.

7. 5. 1. LATEX AGGLUTINATION TEST – CARBOHYDRATE ANALYSIS

Conflicting reports exist about the antigenic activity of polysaccharides from *M. mycoides* subsp. *mycoides* SC. In this thesis, section 5.2.4., polysaccharides were extracted from culture media supernatant following the growth of *M. mycoides* subsp. *mycoides* SC. A modification of a method described by Rurangirwa *et al.* (1987b) was used. Following separation through a BioSep Sec 300 column, it was discovered using an ELISA test that some fractions were more immunogenic than others. The most immunogenic fraction was used for binding to latex beads. By standardising the concentration of antigen and sera used in the LAT the test appeared quite specific. However, it was important to understand the nature of this antigen. Initial analysis of the carbohydrate was carried out using TLC, however this was not conclusive or quantitative, although it indicated the presence of either mannose or fucose, glucosamine and possibly galactosamine. Further analysis was carried out by BioMed Laboratories, (see reports in Appendix 3). This showed that fucose, glucosamine and galactose were present in the ratio 1:2:16 respectively. A sialic acid, *N*-acetyl neuraminic acid was also detected. This is similar to the findings of Plackett *et al.* (1963), but contrasts greatly with the findings of March *et*

al. (1999) who reported that the capsule was composed of *N*-acetylglucosamine, fructose, fucose, glucosamine, glucose and mannose in approximately equal concentrations. The material analysed by March *et al.* (1999) was similar to that obtained in this study when uninoculated medium was analysed. This suggests that the carbohydrate reported by March *et al.* (1999) was in fact derived from a media component, possibly yeast extract; the yeast cell wall contains a polysaccharide known to be rich in glucosamine and *N*-acetylglucosamine. The exact structure of the polysaccharide was not determined in this study, but may be similar to the disaccharide repeating unit β-D-galactofuranosyl-(1-6)-D-galactopyranose described by Smith (1984), although additional carbohydrates were obtained in this study. It would be interesting to confirm this structure. An artificial polysaccharide could then be made and used to develop and detect an immune response to CBPP. It has potential as both a diagnostic antigen and a possible vaccine candidate, although it might induce an auto-immune response because of its similarity to bovine pneumogalactan.

Rice and Miles, (reported by Nicholas *et al.*, 2000a) showed that there was little variation in capsule production between strains but most capsule was produced in the early phases of growth. However, Rice *et al.* (2000) described media (PRM) formulates specifically for the growth of *M. mycoides* subsp. *mycoides* SC that gave high growth rates and yields. The use of such a medium may give increased levels of carbohydrate production and recovery.

7. 6. CONTROL STRATEGIES

Control of *M. bovis* and CBPP by antimicrobials is unlikely, although strategic treatment might have some benefit. Generally in the UK prophylactic treatment is not recommended as it is considered more likely to give rise to antimicrobial resistance (Anon, 1999). However, in theory prophylactic treatment at critical times, at say 6 weeks, when maternal antibodies are declining, and also when introducing new stock to a farm may be beneficial in disease control. This treatment may cause a significant reduction in the mycoplasma population, at the same time giving the animals more time to develop their own immune responses. Such treatment may also lower or mask the detectable immune response in serological

tests, so other control measures can not be taken. The clinical symptoms and general health of *M. bovis* affected cattle may be improved by ensuring good husbandry practice, such as improving housing conditions, thus ensuring the housing is dry with plenty of ventilation, and not mixing older affected animals with younger calves. Depopulation of stock, is the only effective solution to controlling *M. bovis* and CBPP. Where new or replacement stock is being acquired, suitable quarantine and testing procedures may prevent the introduction of disease into the herd. This approach was recently successfully carried out in Ireland (O'Farrell *et al.*, 2001).

From the information obtained in this thesis (chapter 3) the use of antimicrobials in treating CBPP may be supported. However, when compared with the MIC values obtained for *M. bovis* where resistance has developed it is difficult to justify the widespread use of antimicrobials, which may increase the development of antimicrobial resistance. However, *in vitro* testing is only a small part of the overall picture and the pharmokinetics of the antimicrobials should be taken into consideration. Even though some antimicrobials are concentrated many times within the animal (Reeve-Johnson, 1999) such widely disseminated organisms must be hard to attack. When CBPP has affected the lung causing necrosis and the formation of sequestra as seen in chapter 4, antimicrobials are very unlikely to be effective (Provost *et al.*, 1987). Consequently the choice of antimicrobial to treat cases of CBPP should not be based solely on the basis of *in vitro* tests. However, future use of antimicrobials should not be discounted as new improved antimicrobials, particularly derivatives of the fluoroquinolones, such as enrofloxcin, a carboxylic acid derivative (Barragry, 1994) and advocin (see chapter 3) are continually being developed.

The current vaccine for CBPP uses live *M. mycoides* subsp. *mycoides* SC (see section 1.5.5.1.) which has many problems, such as the transient nature of protection, severe adverse reactions at inoculation sites and even difficulties ensuring sufficient dose levels of vaccine are given. There is no vaccine for *M. bovis*. Effective control is limited to removal of affected animals or whole herds. Early identification of affected animals by diagnostic tests is currently essential to prevent spread of these diseases. Development of new and improved vaccines is also required to control these diseases.

7. 7. FURTHER WORK

A major aim of this thesis was the development of improved diagnostic tests that could be used to diagnose two major mycoplasma bovine diseases of cattle. This has been achieved with the routine use of PCR for *M. bovis* and the development of the LAT. Much interest has been shown in the LAT, because of its potential application particularly to the African CBPP crisis where rapid results can be obtained in the field. Under laboratory conditions the test was shown to be as effective as the much more laborious CFT, which is strictly a laboratory-based test. The LAT now needs further development for field use. This would entail re-evaluating the LAT with more whole blood samples, and possibly binding the antigen to black latex beads to make interpretation of the test easier. In the laboratory the test works well with sera and the latex beads can be successfully dried onto a card. However, in the warmer African climate, larger volumes of blood or sera may be needed to reduce drying out of the latex beads-blood/serum mixture. The shelf life of LAT reagents, particularly if applied to cards, also needs to be critically evaluated. The first batch of LAT reagents have been stored at 4°C for more than two years and still work effectively, suggesting a long shelf life for the product if properly stored. The LAT also needs to be evaluated against CBPP vaccinated cattle.

Improved understanding of the LAT antigen would also help increase confidence in the test and possibly enable improvements in its preparation. The antigen was derived from the carbohydrate fraction of culture supernatants that proved most effective in initial LAT studies. It was presumed that culture supernatant carbohydrates were essentially capsular carbohydrates. Analysis of the carbohydrate showed that it was similar to the capsular material described by Plackett *et al.* (1963). The LAT antigen was treated with proteinase to ensure no contaminating proteins were present. Thus, the results obtained in this thesis suggest that the secreted *M. mycoides* subsp. *mycoides* SC polysaccharide is antigenic. Conflicting evidence for polysaccharide antigenicity has been presented previously (see section 5.1.1.) with different fractions containing polysaccharides, possibly proteins and or lipids and heat labile factors. The fractions were also tested by a variety of serological methods that included; immunodiffusion, precipitin tests,

and complement fixing tests. However, the number of conflicting reports published suggest extraction methods are critical to obtaining a pure antigenic polysaccharide. To ensure reproducibility of the LAT standardisation of production methods for the antigenic polysaccharide will be essential. Improvements in methodology are required, to ensure similar, ideally improved growth yields, and to reduce and simplify the stages in the current purification method. The purity of the active antigen and the standardisation of concentrations would all benefit from additional investigation. Use of other culture media such as PRM may increase the yield of organism. A separate study on the effect of medium composition on capsular polysaccharide as a proportion of cell proteins is also worth investigation. Providing the polysaccharide structure is fully determined, a synthetic polysaccharide would probably be more cost effective to produce and easier to standardise. It would also be possible to develop a multi-disease LAT. For example, providing a specific antigen was identified and purified, say for *M. bovis* it could be bound to different coloured latex beads, so both CBPP and *M. bovis* could be tested for with one test. The colour of beads clumping would determine which disease was detected.

In addition to developing the LAT for *M. mycoides* subsp. *mycoides* SC a number of other diagnostic methods were used in this thesis. Clearly, there are always ways to improve existing tests. For example the CFT was modified to use less reagents (Tittarelli *et al.*, 1999). The ELISA tests can always be improved by using better-purified antigens or cocktails of antigens, or for the cELISA different Mabs could be used, or even multiple Mabs. Different Mabs could also be used for the ICC. The immunoblotting method can be improved. The Portuguese test (Gonçalves *et al.*, 1998) uses sera diluted at 1 in 5, which produces many non-specific bands on the immunoblot that require critical interpretation, whereas Nicholas *et al.* (1996) used sera diluted at 1 in 50, so only major bands were detected. The dilution at which a serum is tested can be further optimised. Ideally the five major proteins detected by immunoblotting for CBPP could be purified and only those used on a membranes, thereby giving clearly defined results with a high concentration of sera for maximum sensitivity.

In this thesis a PCR is described that was introduced into routine use in laboratory for the identification of *M. bovis*. This test also differentiates a closely

related exotic pathogen, *M. agalactiae.* The sensitivity of PCRs can be improved. Ideally the test should be sensitive enough to detect a single organisms in clinical samples and nasal swabs. The development of thermocyclers in recent years has increased the speed of PCR, and the introduction of light cyclers (Applied Biosystems, UK) will further increase the speed of PCRs and reduce the volume of reagents and samples required for testing. One difficulty with PCR, is that it detects DNA, therefore one cannot be sure if the organism detected is still viable, which may be important in diagnosing disease if animals have been vaccinated, or successfully treated with antimicrobials. Reverse transcriptase PCR (Newton and Graham, 1994) which detects RNA, may therefore have a role in diagnosis.

Interpretation of MIC values is difficult, and MIC cut-off points do not necessarily relate to effectiveness *in-vivo.* Further investigations of the pharmokinetics of antimicrobials in animal species are required. In this study the MIC cut-off points recommended by Ter Laak *et al.* (1993) have been used. In this thesis widespread antimicrobial resistance within the British *M. bovis* population has been reported. It is therefore important to monitor the further development and spread of resistance, partly to target treatment by using effective antimicrobials. It is also important to assess the risk of potential antimicrobial resistance entering the food chain, either from the accumulation of antimicrobials in the animal, or from the exchange of genetic resistance mechanisms between microorganisms. There is a need to test more antimicrobials to determine the wider extent of resistance to mycoplasmas. It would also be of interest to test 'archived' *M. bovis* strains to see how long it has taken for antimicrobial resistance to develop. The introduction of routine screening methods, such as the method used in this thesis, or possibly using 'E tests' (Cambridge Diagnostic Services, Cambridge). E tests are a quantitative agar method based on the transfer of a continuous concentration gradient of an antimicrobial agent from a plastic strip into an agar medium (Waites *et al.,* 1998). Whilst the basis of some antimicrobial resistance mechanisms are known, such as gene mutations and the presence of plasmids for antimicrobial resistance. Modes of antimicrobial resistance in mycoplasmas and transfer of resistance between mycoplasmas and other microorganisms also require further investigation.

This thesis has only been directly concerned with informed diagnosis and the use of antimicrobials in disease control. However, vaccines are important in disease control. Currently there are no vaccines for *M. bovis*. Several attempts have been made to produce such a vaccine, but none have been successfully marketed. Several trials of different potential *M. bovis* vaccines have shown that they have enhanced the disease (Bryson *et al.*, 1999; Rosenbusch, 1998). A vaccine for CBPP is in use in Africa (see section 1.5.5.1.), however it has many problems including that it is live and provides only transient protection. There is clearly a need for a more effective vaccine, which is stable, risk free, cheap and gives long-term protection.

Possibly, as the whole genome sequences for mycoplasmas become available it will be easier to determine pathogenicity factors and unique features of pathogenic species. They may in time enable identification of potential targets for development of new antimicrobials and protein or other gene products which may be suitable for use in sub-unit vaccines or to which immunity might be gained using DNA vaccines. It might also be possible to develop attenuated vaccine strains by the deletion of pathogenic determinants. Currently, sequencing of the *M. mycoides* subsp. *mycoides* SC genome is almost complete (Persson *et al.*, 2000), however, no attempt has yet been made to sequence the *M. bovis* genome.

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APPENDIX 1

A1. IDENTIFICATION OF MYCOPLASMA SPECIES USING 16S rRNA SEQUENCING

A1. 1. 1. INTRODUCTION

Amplicons produced by PCR using universal primers for the 16S rRNA gene can be sequenced and the sequence used to identify an organism by comparing with others on the databanks. A sequence comparison giving a similarity of over 98% is required to indicate significant homology with another sequence; values lower than 98% would generally indicate a different species (Johansson *et al.*, 1998).

A1. 1. 2. ISOLATES USED FOR 16S rRNA SEQUENCING

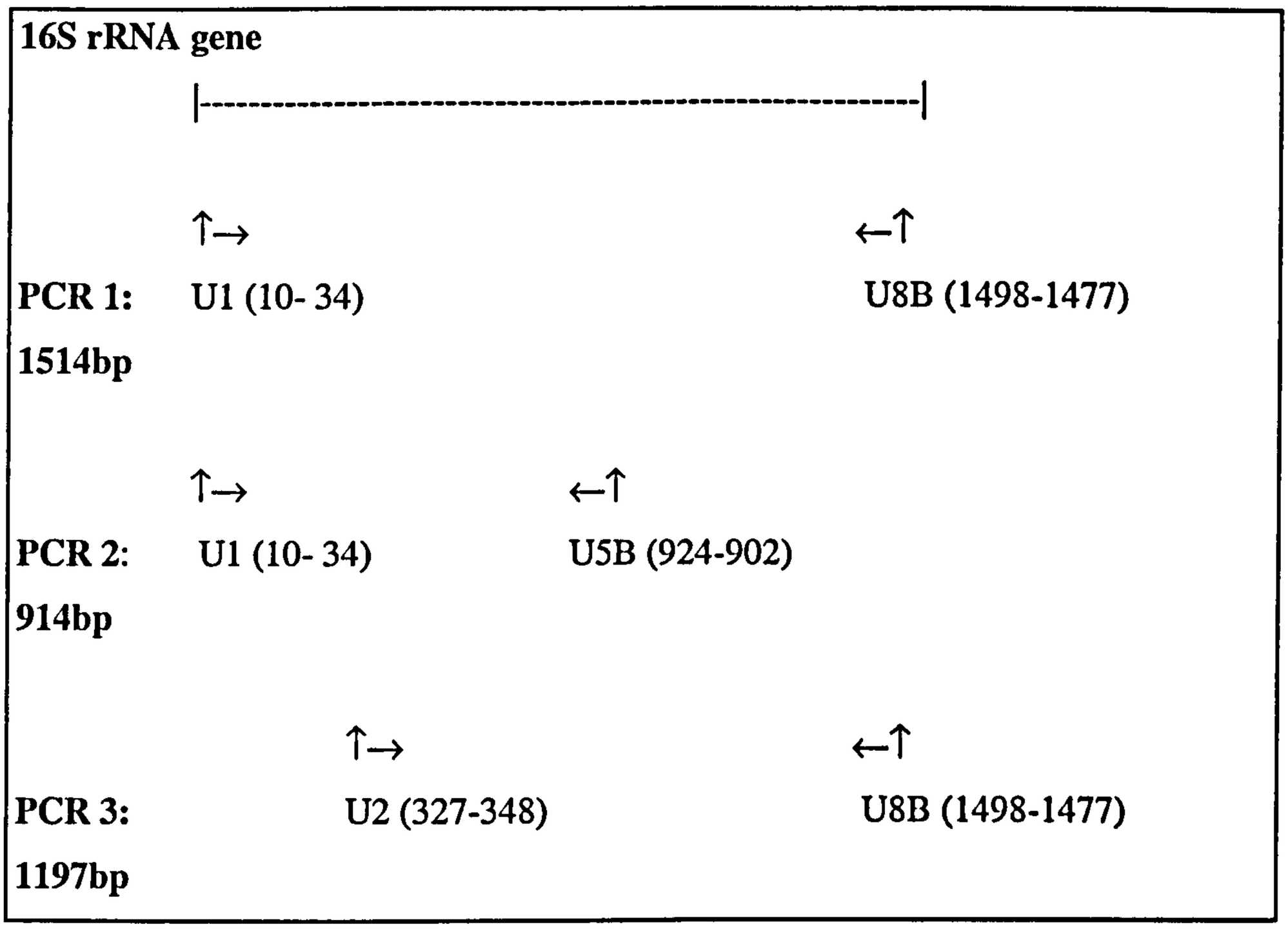
The 16S rRNA gene was sequenced for two mycoplasma isolates that could not be identified using conventional culture and serological tests. One isolate, reference 53V95, came from a peregrine falcon (*Falco peregrinus*) suffering eye lesions submitted to VLA (Weybridge) from VLA Starcross; the other, reference 67A96, had been isolated at VLA (Weybridge) from a cloacal swab taken from a healthy ostrich (*Struthio camelus*) submitted for export testing.

A1. 1. 3. 16S rRNA UNIVERSAL PCR

The strategy for sequencing the 16S rRNA genes requires the use of universal primers. Primers corresponding to the U1 and U8 regions of the 16S rRNA gene were used in a first PCR to give a 1514 base pair (bp) product. This then provided the template for the second PCR reactions using semi-nested primers U1 to U5 and U2 to U8 (fig. A1. 1.) resulting in amplicons of 914bp and 1197bp, respectively. The U5 and U8 reverse primers were biotinylated.

FIGURE A1. 1. UNIVERSAL PRIMER BINDING POSITIONS TO THE 16S rRNA GENE

The numbers (standard typeface) refer to the DNA base sequence according to the numbering of the International Union of Biochemistry for the 16S rRNA gene of *Escherichia coli* (Brosius *et al.*, 1978). The sizes of amplicon (bp) are given in bold typeface.



A1. 1. 4. 16S rRNA SEQUENCING

The method used for sequencing was exactly as described by Johannsson *et al.* (1998). The biotinylated PCR products were immobilised onto streptavidin-coated paramagnetic beads (Dynabeads M280; Dynal AS, Oslo, Norway) and the strands were separated using a standard protocol. Both strands were used for sequencing. The sequencing primers were labelled with Cy5, (Cy5 Auto Read Sequencing Kit, Pharmacia) for detection with the red laser-based automated laser fluorescent DNA sequencing system ALF*express* (Pharmacia). One pmol of primers were used in the sequencing reactions. Clean plates were assembled into a cassette. The gel was poured from ready prepared six % acrylamide solutions and allowed to polymerise for three hours before placing on the automated laser fluorescent DNA sequencer (ALF, Pharmacia LKB Biotechnology AB, Sweden). The temperature was raised to 55°C and the laser allowed to reach optimum conditions before 5 µl of each Sanger sequencing reaction was loaded. The ALF DNA sequencer was then run at 1500V, 60 mA, 25 W for 10 hours at 55°C. Sequences were recorded automatically.

A1. 2. SEQUENCE ANALYSIS

The sequences were aligned and compared by using the program package for sequence analysis from the Genetics Computer Group at the University of Winconsin, USA (Devereux *et al.*, 1984).

A1. 3. RESULTS OF DNA SEQUENCING FOR FALCON AND OSTRICH ISOLATES

The 16S rRNA gene was sequenced for both the falcon and ostrich isolates. A total of 1457 bases were sequenced from the falcon isolate and 1347 bases from the ostrich isolate. As bidirectional sequencing was used, the sequences required manipulation, i.e. reversing, aligning and joining to produce the sequence for the majority of the gene. Data from overlapping segments meant that the majority of the gene was in effect sequenced twice. The sequences obtained from the falcon had seven bases that were unresolved and the ostrich had six; these occurred near the ends of the sequence where errors are most frequent (K-E Johansson, personal communication). Unresolved bases were allocated a code letter suggesting the likely base(s), details are given in Table A1. 1.

TABLE A1. 1. CODE LETTERS FOR UNRESOLVED BASES IN THE SEQUENCES REPORTED IN FIGURES A1. 2 and A1. 3

CODE	M	R	W	S	Y	K	V	H	D	B	N
POSSIBLE BASES	AC	AG	AT	CG	CT	GT	AC G	AC T	AG T	CG T	AC GT

FIGURE A1. 2. THE DNA SEQUENCE OF THE 16S rRNA GENE FROM THE PEREGRINE FALCON ISOLATE

```
GTGTGC*YTAA TACATGCATG TCGAGCGGAG TTCT*YCGGAA  40
CTTAGCGCCG AATGGGTGAG TAACACGTAC TTAACGTACC  80
TCTTAGATTG GGATAACGGT GAGAAATCAC CGCTAATACC  120
GGATACTTAT AAAGA*VGGCA TCTTCTTTAT ATAAAAGGAG  160
CTCTCAAGCT CCACTAGGAG ATCGGGGTGC GGAACATTAG  200
TTAGTTGGTG AGGTAATGGC TCACCAAGAC TATGATGTTT  240
AACGGGGTTG AGAGACTGAT CCGTCACACT GGGACTGAGA  280
TACGGCCCAG ACTCCTACGG GAGGCAGCAG TAGGGAATTT  320
TCCACAATGG GCGAAAGCCT GATGGAGCGA CACAGCGTGC  360
AGGATGAAGG CCTTCGGGTT GTAAACTGCT GTTATTTAGG  400
ATGAAAAAAT AGTAGAGGAA ATGCTATTAT CTTGACAGTA  440
CTAAATCAGA AAGCAACGGC TAACTATGTG CCAGCAGCCG  480
CGGTAATACA TAGGTTGCAA GCGTTATCCG GAATTATTGG  520
GCGTAAAGCG TCTGTAGGTT GTTTATTAAG TCTGGCGTCA  560
AAACCTTGGG GCTCAACCCC AAATCGCGTT GGATACTGAT  600
AAACTAGAAT TGTGTAGAGG TTAACGGAAT TCCTTGTGAA  640
GCGGTGAAAT GCGTAGATAT AAGGAAGAAC ATCAACTTGG  680
CGAAGGCAGT TAACTGGGCA CATATTGACA CTGAGAGACG  720
AAAGCGTGGG GAGCAAACAG GATTAGATAC CCTGGTAGTC  760
CACGCCGTAA ACGATGATGA TTAGCTGATG GGAACCATCG  800
GCGCA*STAAC GCATTAAATC ATCCGCCTGA GTAGTATGCT  840
CGCAAGAGTG AAACTTAAAG GAATTGACGG GGATCCGCAC  880
AAGCGGTGGA GCATGTGGTT TAATTTGAAG ATACGCGTAG  920
AACCTTACCC ACTCTTGACA TCTTCTGCAA AGCTATAGAG  960
ATATAGTGGA GGTTAACAGA ATGACAGATG GTGCATGGTT  1000
GTCGTCAGCT CGTGTCGTGA GATGTTCGGT TAAGTCCTGC  1040
AACGAGCGCA ACCCTTGTCC TTAGTTAAAT ATCCTAAGGA  1080
GACTGCCCGG GTAACTGGGA GGAAGGTGGG GACGACGTCA  1120
AATCATCATG CCTCTTACGA GTGGGGCAAC ACACGTGCTA  1160
CAATGGACGG TACAAAGAGA AGCAATATGG CGACATGGAG  1200
CAAATCTCAA AAAACCGTTC TCAGTTCGGA TTGTAGTCTG  1240
CAACTCGACT ACATGAAGTC GGAATCGCTA GTAATCGTAG  1280
ATCAGCTACG CTACGGTGAA TAACGTTCTC GGGGTCTTGT  1320
ACACACCGCC CGTCACACCA TGGGAGCTGG TAATGCCCGA  1360
AGTCGGTTTA GTCAACTACG GAGACAACTG CCTAAGGCAG  1400
GACTGGTGAC TGGGGTGAAG TCGTAACAAG GTATCCCTAC  1440
GAGAACGT*RC GGAT*R*RA  1457
```

* Unresolved bases code shown in Table A1.1.

FIGURE A1. 3. THE DNA SEQUENCE OF THE 16S rRNA GENE FROM THE OSTRICH ISOLATE

```
T*B*BGCATGAA GTTAATATAA AAGGAGCGTT TCTTCGCTAG   40
AGGATCGGGG TGCGTAACAT TAGTTAGTTG GTGAGGTAAT   80
GGCTCACCAA GACGATGATG TTTAGCGGGG TTGAGAGACT   120
GATCCGCCAC ACTGGGACTG AGATACGGCC CAGACTCCTA   160
CGGGAGGCAG CAGTAGGGAA TTTTCCACAA TGGGCGAAAG   200
CCTGATGGAG CGACACAGCG TGCGCGATGA AGGCCTTCGG   240
GTTGTAAAGC GCTGTTATAA GGGAAGAAAA AGCAGTAGAG   280
GAAATGCTAT TGCCTTGACG GTACCTTGTC AGAAAGCAAC   320
GGCTAACTAT GTGCCAGCAG CCGCGGTAAT ACATTAGGTT   360
GCAAGCGTTA TCCGGAATTA TTGGGCGTAA AGCGTCTGTA   400
GGTTGTTTGT TAAGTCTGGT GTGAAAACTT GGAGCTCAAC   440
TCCAAATTGC ATTGGATACT GGCAAACTAG AGTTATGTAG   480
AGGTAAGCGG AATTCCTTGT GAAGCGGTGG AATGCGTAGA   520
TATAAGGAAG AACACCAACC TGGCGAAGGC AGCTTACTGG   560
ACATATACTG ACACTGAGAG ACGAAAGCGT GGGGAGCAAA   600
CAGGATTAGA TACCCTGGTA GTCCACGCCG TAAACGATGA   640
TGATTAGCTG ATGGGGAGCT CATCGGCGCA *STAACGCATT   680
AAATCATCCG CCTGAGTAGT ATGCTCGCAA GAGTGAAACT   720
TAAAGGAATT GACGGGGATC CGCACAAGCG GTGGAGCATG   760
TGGTTTAATT TGAAGATACG CGTAGAACCT TACCCACTCT   800
TGACATCTTC CGCAAAGCTA TAGAGATATA GTGGAGGCTA   840
ACGGAATGAC AGATGGTGCA TGGTTGTCGT CAGCTCGTGT   880
CGTGAGATGT TCGGTTAAGT CCTGCAACGA GCGCAACCCT   920
TGTCCTTATT TAGATGATAT AAGGAGACTG CCCGGGTAAC   960
TGGGAGGAAG GTGGGGACGA CGTCAAATCA TTCATGCCTC   1000
TTACGAGTGG GGCAACACAC GTGCTACAAT TGGACGGTAC   1040
AAAGAGAAGC AATACGGCGA CGTTGAGCAA ATCTCAAAAA   1080
ACCGTTCTCA GTTCGGATTG TAGTCTGCAA CTCGACTACA   1120
TGAAGTCGGA ATCGCTAGTA ATCGTAGATC AGCTACGCTA   1160
CGGTGAATAC GTTCTCGGGT CTTGTACACA CCGCCCGTCA   1200
CACCATGGGA GCTGGTAATG CCCGAAGTCG GTTTTGTTAA   1240
CTACGGAGAC AACTGCCTAA GGCAGGACCG GTGACTGGGG   1280
TGAAGTCGTA ACAAGGTATC CCTACGAGAA CGTGCGG*RTG   1320
GA*W*HACCTCC TTTCGGTCAT AGCTGTT   1347
```

* Unresolved bases code shown in Table A1. 1.

A1. 4. DNA SEQUENCE ANALYSIS

Sequence comparisons showed the best fit with 50 mycoplasma species, indicating the isolate belonged to the mycoplasma group. Details of the closest best-fit comparisons are given in Table A1.2.

TABLE A1. 2. BEST FIT COMPARISONS FOR THE PEREGRINE FALCON AND THE OSTRICH 16S rRNA SEQUENCE

PEREGRINE FALCON		**OSTRICH**	
SPECIES	***% SIMILARITY***	***SPECIES***	***% SIMILARITY***
M. gallinaceum	96.1%	*M. gallinaceum*	94.6%
M. coragypsum	94.3%	*M. coragypsum*	93.4%
M. leocaptivus	94.0%	*M sturnidae*	92.9%
M. sturnidae	93.8%	*M. leocaptivus*	92.5%
M. gallinarum	93.3%	*M. synoviae*	92.0%
M. bovis (Donetta)	93.2%	*M. gallinarum*	91.8%
M. lipophilum	92.9%	*M. meleagridis*	91.5%
M. meleagridis	92.8%	*M. arginini*	87.3%
M. synoviae	92.8%		
M. fermentans	92.8%		
M. agalactiae	92.7%		
M. felifaucium	92.6%		
M. californicum	92.4%		
M. bovigenitalium	92.3%		
M. simbae	91.3%		
M. leopharyngis	92.0%		
M. gatae	89.3%		
M. hyopneumoniae	87.9%		

For the peregrine falcon no identical sequence was found and the highest similarity (96.1%) was with *M. gallinaceum.* Of the 1457 bases sequenced, 423 were A (29.03 %), 400 were G (27.45 %), 337 were T (23.13 %), and 290 were C (19.90 %); 7 were unresolved (0.48 %).

Like the peregrine falcon isolate, no identical sequence was found for the ostrich. Of the 1347 bases sequenced, 373 were A (27.69 %), 387 were G (28.73 %), 310 were T (23.01 %), and 271 were C (20.12 %) and 6 were unresolved (0.45 %).

Comparison of the sequences obtained for the ostrich and falcon isolates gave a 94.5% similarity showing that they are different *Mycoplasma* species.

A1. 5. DISCUSSION

Knowledge of the pathogenicity of the isolates would also be useful. Interestingly, several isolates from ostriches have since been submitted to the VLA, all of which reacted with the antisera raised against this ostrich isolate. One of these recent isolates came from a 13-14 week old ostrich from a group of 12 birds, which were sneezing and coughing; some also had a white or clear nasal discharge. They had all lost weight and failed to respond to antimicrobial treatment with enrofloxacin and oxytetracycline. Three birds died but only one was examined. The sinuses contained a clear mucoid content but no gross abnormalities of the trachea, lungs or air sacs was seen and the cause of death was not established. Thus, the mycoplasma may be associated with disease. The isolation of a potential pathogen from ostriches is commercially important as the number of ostrich farms is increasing to meet export demands and requirements for alternative foods.

Since this work was done 16S rRNA sequencing has also been successfully used to identify a number of unusual isolates submitted to VLA (Weybridge). *M. fermentans* was identified from a sheep (Nicholas *et al.,* 1998); *M. auris* from a Nepalese goat; isolates from penguins have been shown to have sequences similar to the avian species, *M. gallisepticum* and *M. imitans.* A commercial kit MicroSeq (Perkin Elmer) has recently been marketed to aid bacteria identification that provides all the necessary reagents to carry out PCR and cycle sequencing reactions. However, in preliminary trials, not all mycoplasmas were amplified by the PCR from this kit.

The 16S rRNA sequencing is a useful method that aids identification of unusual isolates or indicates the possibility of new species being isolated. Although this study used isolates from non-bovine hosts it may also be useful for mycoplasma isolates from cattle, including those associated with bovine pneumonia. The identification of the two isolates sequenced here is continuing but is outside the scope of this project.

APPENDIX 2

MIC AND MMC VALUES

TABLE A2. 1. MIC AND MMC VALUES FOR STRAINS OF *M. BOVIS* (STRAINS 1-62) AND *M. MYCOIDES* SUBSP *MYCOIDES* SC (STRAINS 63-82)

All values are given in μg/ml.

	OXYTET		SPECT		FLOR		TILM		DANO	
STRAIN	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC
1	16	64	4	4	4	16	>128	>128	0.25	0.25
2	32	64	8	8	8	32	>128	>128	0.25	0.5
3	64	128	>128	>128	4	16	>128	>128	0.5	0.5
4	64	64	8	>128	4	8	>128	>128	0.5	1
5	8	16	4	4	16	16	>128	>128	0.5	0.5
6	32	64	>128	>128	2	16	>128	>128	0.5	0.5
7	16	128	4	8	16	32	>128	>128	0.25	0.5
8	2	64	4	32	4	16	>128	>128	0.25	0.5
9	32	128	4	32	4	32	>128	>128	0.25	0.25
10	32	128	4	64	4	32	>128	>128	0.5	2
11	16	128	4	32	32	64	>128	>128	0.25	0.5
12	32	32	4	16	8	8	128	>128	0.25	0.5
13	32	64	2	16	8	8	>128	>128	0.25	0.5
14	32	64	2	2	4	32	>128	>128	0.5	0.5
15	32	64	4	32	8	64	>128	>128	0.25	0.5
16	2	2	8	8	8	8	>128	>128	0.5	0.5
17	32	64	4	4	64	128	>128	>128	0.25	0.5
18	4	4	8	16	8	8	4	16	0.25	0.5
19	1	32	4	8	2	16	>128	>128	0.25	0.25
20	32	32	4	4	32	32	>128	>128	0.5	0.5
21	128	128	4	8	8	16	>128	>128	0.5	0.5
22	32	64	>128	>128	2	16	>128	>128	0.5	0.5
23	16	64	2	16	16	32	>128	>128	0.5	0.5
24	32	64	4	8	4	16	>128	>128	0.5	0.5
25	16	32	8	8	64	32	>128	>128	0.25	0.5
26	16	64	>128	>128	4	16	>128	>128	0.5	0.5
27	4	64	2	64	8	32	>128	>128	0.25	4
28	16	>128	>128	>128	4	16	>128	>128	0.5	1
29	32	32	>128	>128	8	16	>128	>128	0.5	0.5
30	32	64	2	4	4	16	>128	>128	0.25	0.5
31	64	>128	8	128	4	32	>128	>128	1	4
32	32	128	>128	>128	16	16	>128	>128	0.5	0.5
33	32	128	4	16	1	8	16	>128	0.5	8
34	81	128	4	4	4	16	>128	>128	0.5	0.5
35	64	64	>128	>128	16	16	>128	>128	0.5	0.5
36	8	32	8	8	2	8	>128	>128	0.125	0.5
37	16	32	4	4	32	32	>128	>128	0.25	0.5

STRAIN	OXYTET MIC	OXYTET MMC	SPECT MIC	SPECT MMC	FLOR MIC	FLOR MMC	TILM MIC	TILM MMC	DANO MIC	DANO MMC
38	32	64	4	4	4	16	4	32	0.5	0.5
39	8	32	4	32	4	16	128	>128	0.5	0.5
40	8	32	1	8	1	8	8	32	0.25	0.5
41	4	16	4	4	4	8	>128	>128	0.5	0.5
42	64	64	>128	>128	4	32	>128	>128	0.5	0.5
43	32	64	4	4	4	32	>128	>128	0.25	0.5
44	32	64	128	>128	8	16	>128	>128	1	1
45	32	64	2	8	4	16	>128	>128	0.25	0.25
46	8	64	2	2	4	32	>128	>128	0.25	0.25
47	64	64	>128	>128	8	16	>128	>128	1	0.5
48	32	64	4	8	4	16	>128	>128	0.25	0.5
49	32	64	4	16	16	64	128	>128	0.5	0.5
50	16	64	>128	>128	8	16	>128	>128	0.5	0.5
51	16	64	4	>128	16	64	>128	>128	0.5	1
52	32	64	4	4	8	16	>128	>128	0.25	0.5
53	16	32	4	32	4	8	>128	>128	0.25	0.5
54	8	32	2	8	4	16	>128	>128	0.5	0.5
55	16	64	4	4	4	32	>128	>128	0.25	0.5
56	64	128	4	32	8	16	>128	>128	0.25	0.5
57	16	128	2	16	8	32	>128	>128	0.25	0.5
58	4	16	2	8	4	8	>128	>128	0.25	0.5
59	32	64	4	8	8	16	128	>128	0.125	0.5
60	1	2	4	4	4	4	32	32	0.25	0.25
61	16	64	>128	>128	4	16	>128	>128	0.5	0.5
62	1	64	128	>128	1	32	>128	>128	2	2
63	0.25	8	8	32	0.25	4	<0.008	0.03	0.25	0.5
64	0.125	1	4	4	0.5	1	0.015	0.03	0.25	0.5
65	0.125	8	16	64	1	8	0.015	0.06	0.25	0.5
66	1	32	16	16	1	16	0.03	0.125	0.5	0.5
67	0.5	8	8	64	1	16	0.03	0.125	0.25	2
68	0.5	8	8	8	2	8	0.015	16	0.25	0.25
69	4	32	>128	>128	8	16	0.125	0.5	0.5	4
70	0.5	8	8	16	1	8	0.015	0.125	025	0.5
71	0.5	8	8	8	1	8	<0.008	<0.008	0.25	0.5
72	1	1	8	8	1	2	0.015	0.03	0.125	0.25
73	1	8	16	64	1	8	0.015	0.06	0.5	1
74	1	8	8	128	2	16	0.015	0.06	0.5	0.5
75	1	8	8	128	1	8	<0.008	0.015	0.5	4
76	0.5	8	8	16	1	8	0.015	0.25	0.254	0.25
77	0.125	1	8	8	1	4	0.015	0.03	0.25	0.25
78	1	2	8	16	0.5	4	0.03	0.125	0.5	0.5
79	0.5	0.5	16	16	2	1	0.015	0.015	1	2
80	0.5	4	4	8	0.5	1	<0.008	>128	0.25	0.25
81	1	32	16	16	0.5	8	0.03	64	0.5	1
82	1	8	32	64	2	8	0.25	0.25	0.5	0.5

APPENDIX 3

MONOSACCHARIDE COMPOSITIONAL ANALYSIS REPORT 98-01-VLA

'MOVING BIOTECHNOLOGY FORWARD'

MONOSACCHARIDE COMPOSITIONAL ANALYSIS REPORT 98-01-VLA

Prepared for

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December 1998

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Compositional Analysis Report on sample from Veterinary Laboratories Agency

One sample was supplied by the customer in dried form for analysis of neutral monosaccharides. The amount of sample was estimated at 1mg by the customer

Details as supplied by customer

The sample is an extract from media from mycoplasma culture and was prepared for analysis by re-precipitation from water and supplied in lyophilised form. This material has not been hydrolysed. Upon receipt at BioMed labs the sample was stored at –20° before analysis.

Hydrolysis of sample

The total sample was dissolved in 760µl of 0.1M TFA. From this 380µl were removed for acid hydrolysis and as described in the protocol below. Compositional analysis for neutral monosaccharides was then performed on the Dionex carbohydrate analysis system (DX300) against reference monosaccharide standards using a PA 1 column. The remaining solution was stored frozen at –20'

Protocol for analysis

1. Dissolve sample in 380µ10.1M TFA and transfer to an acid-washed tube. Cap the tube and heat for 1h at 100°C in a heating-block for 1 hour.
2. Remove 80µ1 and store at -20°C for analysis of sialic acid if required in the future.
3. Add 300 µ1 of 4M TFA and heat sample for a further 4h at 100°C in a heating-block.
4. Cool tubes and centrifuge at 1500 rpm for 5 min.
5. Freeze sample in liquid nitrogen and lyophilize in freeze-dryer overnight.
6. Dissolve dried material in 250µ1 of water and store frozen until required.
7. Prior to monosaccharide analysis, pass sample through a Microcon filter (10 kD cut off, PVDF)
8. Analyze samples on Dionex machine with PAI column and pulsed amperometric detection at voltage settings recommended by manufacturer for the separation of monosaccharides.
9. Use a flow rate of 1 ml/min of 22 mM NaOH.
10. Set detection at 1000 nA
11. Sample injection volume 5µl
12. Injection sequence standard, sample, blank injection
13. Compare each sample injection with adjacent standard to compensate for any drift in position of peaks.
14. Wash column after each run with 200mM NaOH and re-equilibrate with 22mMNaOH.

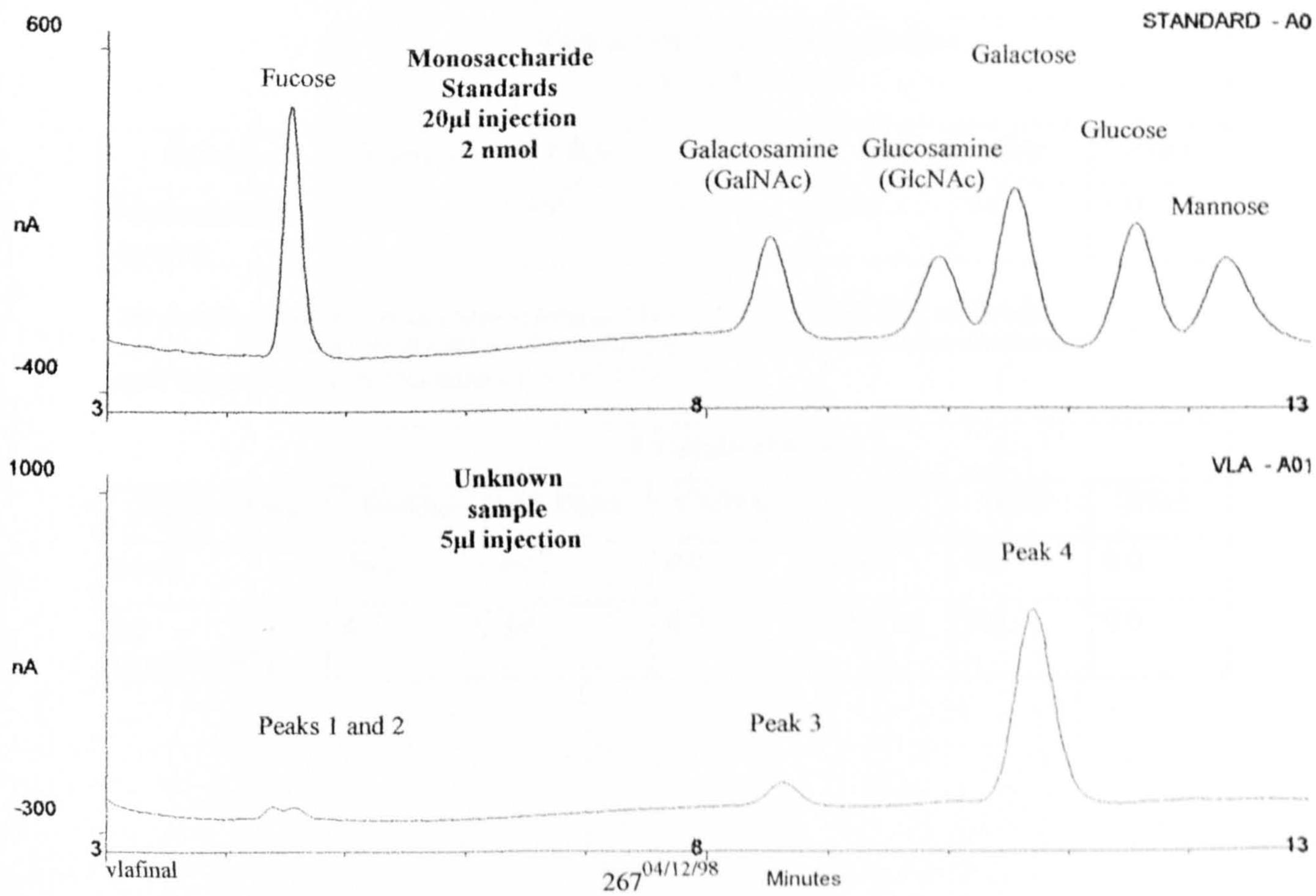
Results of Analysis

Dionex monosaccharide standards used

Monosaccharide	Source	Reference Number	Concentration (nmol /µl)
L Fucose	Dionex	6696-41-9	0.1
α-D Galactosamine	Dionex	1772-03-8	0.1
α-D Glucosamine	Dionex	66-84-2	0.1
α-D Galactose	Dionex	59-23A	0.1
α-D Glucose	Dionex	492-62-6	0.1
α-D Mannose	Dionex	3458-28-4	0.1

The chromatograms obtained from the standard monosaccharides and the samples are shown below.

Analysis of primary monosaccharide standards and extract from mycoplasma culture



Analysis of Results

Standard Monosaccharides

Separation of standards (20 µl) at a concentration of 0.1 nmol/ µl shows elution positions of each monosaccharide.

Extract from mycoplasma

An aliquot of 5µl of the re-dissolved acid hydrolysate was separated. A total of four peaks were detected. Peak 4 is the major peak and corresponds in elution position to galactose. Peak 3 corresponds to the elution position of N-acetyl galactosamine (converted to galactosamine during the acid hydrolysis) . Two further minor peaks were detected. Peak 2 appears to correspond to the elution of fucose but the identity of peak 1 is unknown.

Calculation of amount of monosaccharide in sample

The mean area for each peak of the sample was measured from duplicate runs. Peak areas of monosaccharides were also measured from duplicate standard runs. From these values and the known concentrations of the standards, the concentration of each monosaccharide in the sample was calculated, after adjusting the areas of the standard and the sample for different injection volumes. The monosaccharide concentrations in the sample were as follows:

	Monosaccharide Concentration nmol/µl hydrolysate					
Sample	**Fucose**	**GalNAc**	**GlcNAc**	**Gal**	**Glc**	**Man**
Mycoplasma extract	0.045	0.095	0.0	0.70	0.0	0.0

The sample injected was from a total volume of 250µl . This represents 40% of the total sample i.e. 300µl from 760µl solution. Therefore, the total amount of each monosaccharide in nmol is (monosaccharide concentration) x 250 x 760 / 300.

	Monosaccharide					
Content/1mg	**Fucose**	**GalNAc**	**GlcNAc**	**Gal**	**Glc**	**Man**
nmol	28.7	60.3	0.0	441.9	0.0	0.0
µg (nmol x M^r)	4.7	13	0.0	79.6	0.0	0.0

Conclusions

1. The mycoplasma culture extract contains approximately **9.7%** carbohydrate which is present mainly as fucose, N-acetyl-galactosamine and galactose.

2. **Galactose** is the most abundant of these monosaccharides, representing **about 8%** of dried material that was supplied.

3. Fucose and N-acetyl-galactosamine are present in much smaller amounts, the molar ratio Fuc:GalNAc:Gal being approximately **1:2:15.**

4. Another minor component is present but this would require further work to be identified

5. Sialic acids may also be present. This would not be detectable under the chromatography conditions used for neutral monosaccharides. A suitable aliquot for sialic acid determination was removed prior to the main hydrolysis and this could be used in a future analysis.

Reported dated December 4th , 1998

Prepared by ..... Tony Merry

Checked by ... Graham Turner

BioMed Laboratories , 28, Eastern Way, Ponteland, Newcastle upon Tyne, NE20 9PF.

APPENDIX 3.

MONOSACCHARIDE COMPOSITIONAL ANALYSIS REPORT 99-01-VLA

'MOVING BIOTECHNOLOGY FORWARD'

MONOSACCHARIDE COMPOSITIONAL ANALYSIS REPORT 99-01-VLA

Prepared for
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March 1999

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Compositional Analysis Report on samples from Veterinary Laboratories Agency

Three samples were supplied by the customer in dried form for analysis of neutral monosaccharides. One sample was supplied previously and a neutral monosaccharide analysis performed. It has now been analysed for sialic acid.

Details as supplied by customer

BioMed ref.	Details
VL01	Sample 1 is an extract from media from mycoplasma culture
VL02	Sample 2 is an extract from the harvested cells
VL03	Sample 3 was purified from medium and prepared for analysis by re-precipitation from water , sodium azide was added
VL04	Sample 4 was previously analysed for neutral monosaccharides

Upon receipt at BioMed labs all samples were stored at –20° before analysis.

Hydrolysis of samples

Samples 1-3 were dissolved in 2 M TFA and an appropriate volume removed for acid hydrolysis as described for analysis VLA01-98. Compositional analysis for neutral monosaccharides was then performed on the Dionex carbohydrate analysis system (DX300) against reference monosaccharide standards using a PA 1 column. The remaining solution was stored frozen at –20'. For sialic acid determination on sample 4 hydrolysis was performed in 0.1M TFA at 100°C for two hours.

Results of Analysis

Dionex monosaccharide standards used

Monosaccharide	Source	Reference Number	Concentration (nmol /µl)
L Fucose	Dionex	6696-41-9	0.1
α-D Galactosamine	Dionex	1772-03-8	0.1
α-D Glucosamine	Dionex	66-84-2	0.1
α-D Galactose	Dionex	*59-23A*	0.1
α-D Glucose	Dionex	492-62-6	0.1
α-D Mannose	Dionex	3458-28-4	0.1

The chromatograms obtained from the standard monosaccharides and the samples are shown below.

Figure 1 Analysis of primary monosaccharide standards – typical chromatogram

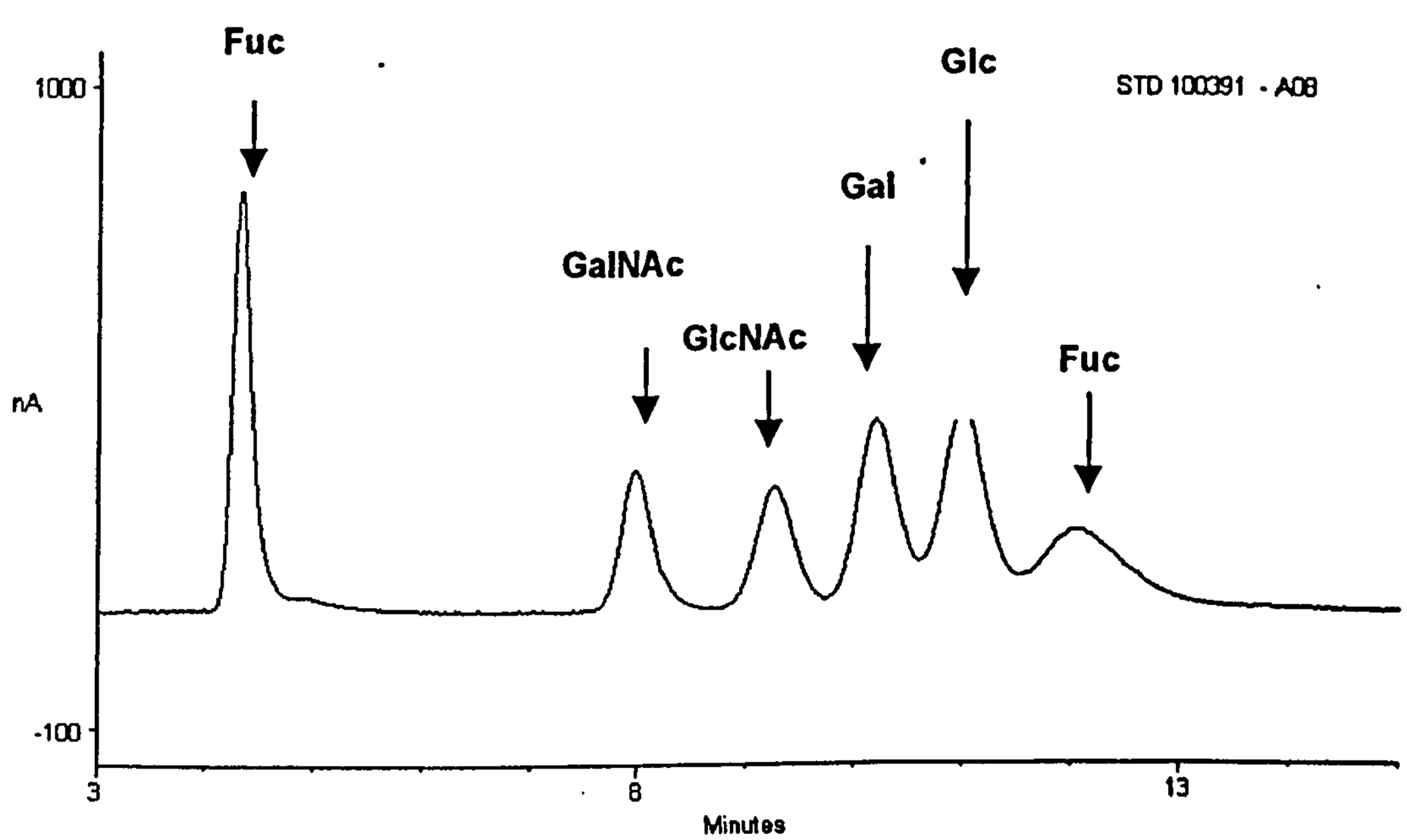


Figure 2 Analysis of sialic acids

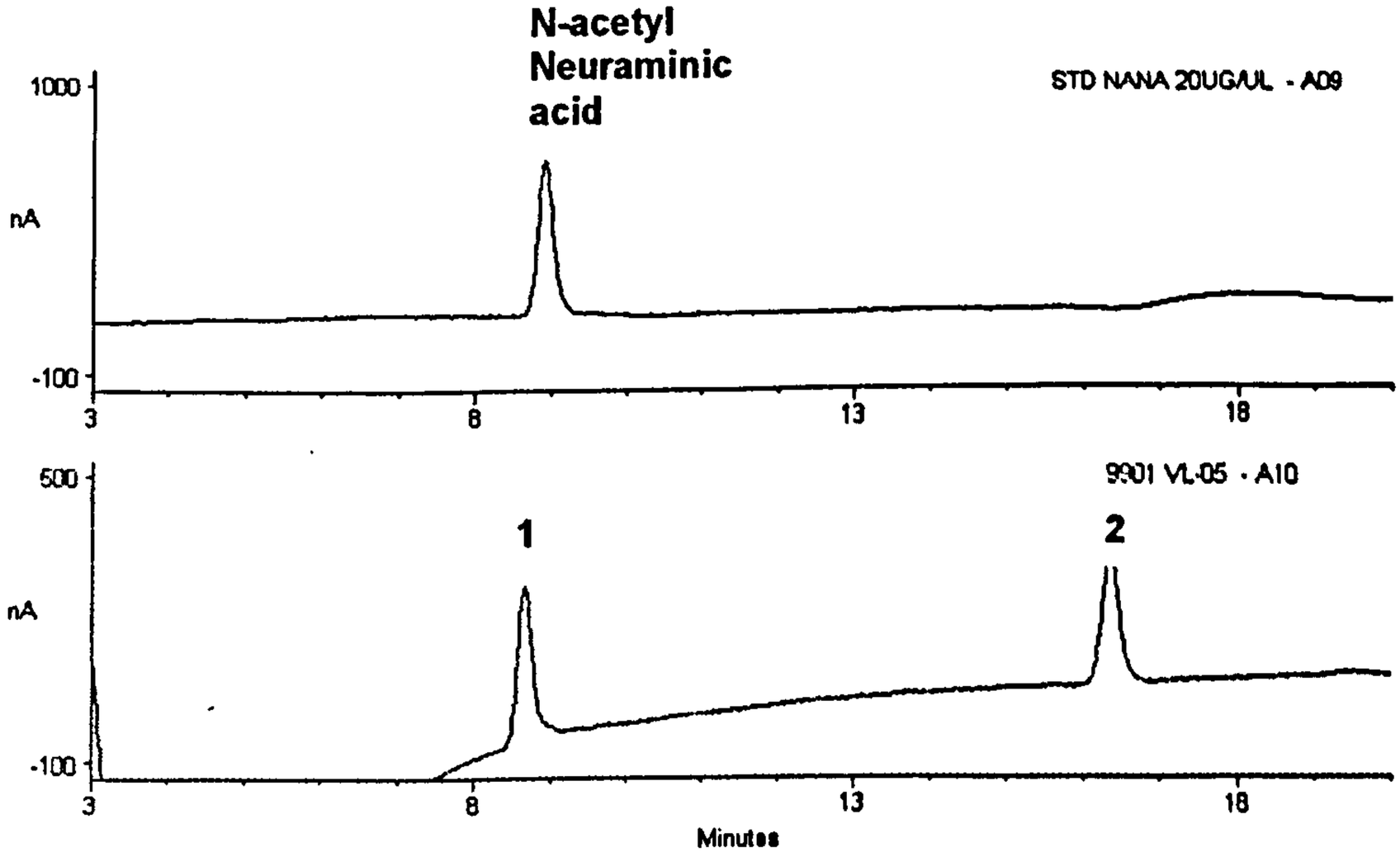
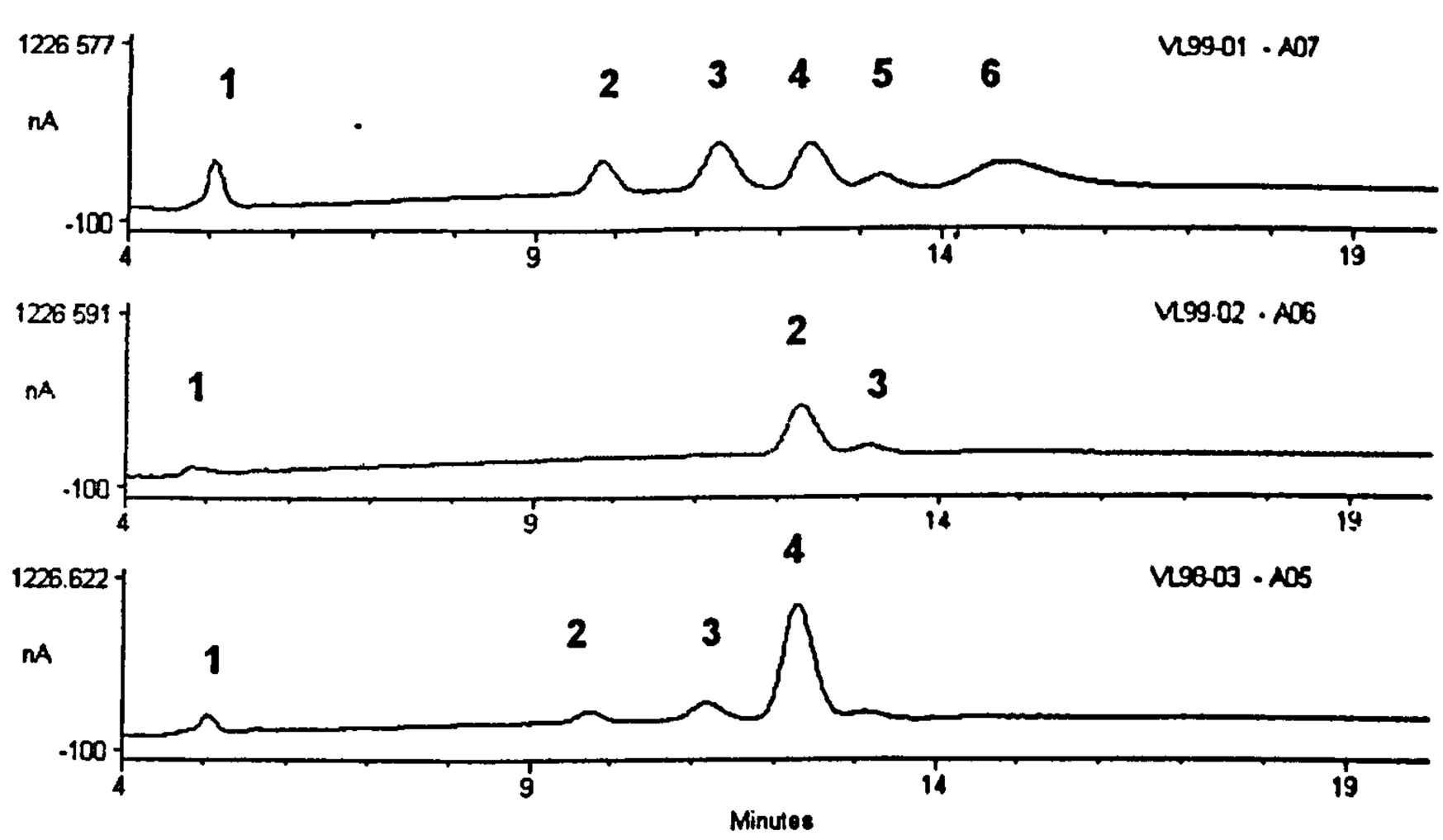


Figure 3 Sample Chromatograms – neutral monosaccharides



Analysis of Results

Standard Monosaccharides

Separation of standards (10 µl injection) at a concentration of 0.1 nmol/ µl Figure 1 shows elution positions of each monosaccharide. This is used to standardise the peak areas and response for each monosaccharide

Samples

1. Medium extract
2. Cell extract
3. Preparation
4. Preparation for sialic acid analysis

Sialic Acid Analysis

Figure 2 shows a chromatogram of a standard preparation of N-acetyl neuraminic acid (20µl injection 20 ng/µl and a corresponding chromatogram of sample 4
From the chromatogram shown in the peak 1 in the sample appears to be N-acetyl neuraminic acid. The second peak could not be identified. Addition of standard N-acetyl neuraminic acid to the sample showed the peak was very close but did not co-elute. Positive identification cannot therefore be made.

Calculation of the concentration in peak 1 by comparison of the area in of the standard peak gives estimated amount in the sample would of 76.75 nmol

Neutral Monosaccharide Analysis

Identification of peaks

From the retention time of the peaks in chromatograms shown in Figure 2 and comparison with that of the standard monosaccharides the following peak identification was made

	Peak Identification					
Sample	**Peak 1**	**Peak 2**	**Peak 3**	**Peak 4**	**Peak 5**	**Peak 6**
VL-01	Fuc	GalNAc	GlcNAc	Gal	Glc	Man
VL-02	Fuc	Gal	Glc			
Vl-03	Fuc	GalNAc	GlcNAc	Gal		

Calculation of amount of monosaccharide in samples

The mean area for each peak of the sample was measured from duplicate runs. Peak areas of the standard monosaccharides were also measured from triplicate standard runs. From these values and the known concentrations of the standards, the amount of each monosaccharide in the sample was calculated

The control sample of medium supplied showed trace amounts of glucose and fucose which were insignificant and probably due to contamination

	Dilution Factor	**Monosaccharide Quantitation nmol**					
Sample	**Sample dilution**	**Fucose**	**GalNAc**	**GlcNAc**	**Gal**	**Glc**	**Man**
VL-01	20/820	184	302	597	287	91	840
VL-02	20/540	26	0	3	176	31	4
VL-03	300/600	8	9	21	68	4	0

	Monosaccharide µg					
Sample	**Fucose**	**GalNAc**	**GlcNAc**	**Gal**	**Glc**	**Man**
VL-01	30.3	65.1	128.8	51.8	16.4	151.3
VL-02	4.3	0	0.7	31.7	5.6	0.8
VL-03	1.3	1.9	4.5	12.5	0.8	0

Conclusions

1. The composition of the media extract (sample 1) is similar to that of serum glycoproteins with N and O linked glycosylation. The total monosaccharide content is **443.8 µg**

2. The composition of the cell extract (sample 2) shows elevated galactose in proportion to the other monosaccharides and is similar to that of the extract studied in report 98-01 VLA The total monosaccharide content is **43 µg** of which **31.7 µg** is galactose

3. The composition of sample 3 also shows a high galactose concentration and the ratios of the other monosaccharides is similar to the extract studied in the previous report. Unlike the previous sample some N-acetylglucosamine was detected. The total monosaccharide content is **22.1 µg** of which **12.5 µg** is galactose.

4. The mycoplasma extract sample analysed in report 98-01 contains sialic acid in the form of N-acetyl neuraminic acid and another unidentified peak. The amount of N-acetyl neuraminic acid is **76.8 nmol**.

Reported dated17 March 1999

Prepared by ..... Tony Merry

Checked by ..... Graham Turner

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APPENDIX 4

RESULTS FROM CBPP AFFECTED FARMS IN PORTUGAL

TABLE A4. 1. COMPARISON OF ACTUAL RESULTS OF SAMPLES FROM CATTLE FROM CBPP AFFECTED FARMS IN PORTUGAL: FARM 1

SAMPLE NO.	LESIONS	LAT	CFT	ELISA	IBT
1	None	-	-	0.24	+
2	Positive	+	1:160	0.62	+
3	Positive	-	1:40	0.74	+
4	Positive	+	1:10	0.78	+
5	None	+	1:10	1.04	+
6	Positive	+	1:20	0.73	+
7	None	+	1:10	0.43	+
8	Positive	+	1:10	0.50	+
9	Positive	+	1:40	0.45	+
10	Positive	+	1:20	0.80	+
11	None	+	1:10	0.53	+
12	None	-	-	0.31	+
13	Positive	-	1:20	0.62	+
14	None	+	-	0.58	+
15	None	-	-	0.27	-
16	Positive	-	1:20	0.61	+
17	None	+	1:80	0.72	+
18	Positive	+	1:40	0.83	+
19	Positive	-	1:40	0.90	+
20	nd	+	1:40	0.62	+
21	Positive	+	1:20	0.69	+
22	None	-	-	0.30	+
23	Positive	+	1:80	1.20	+
24	Positive	+	1:20	0.76	+
25	Positive	-	1:10	0.48	+
26	None	+	1:320	1.22	+
27	Positive	+	1:40	0.44	+
28	None	+	1:10	0.44	+
29	None	+	1:10	0.54	-
30	None	+	1:20	0.56	+
31	Positive	-	1:20	0.87	+
32	Positive	+	1:80	0.98	+
33	Positive	+	1:80	0.87	+
34	Positive	+	1:10	1.00	+
35	None	+	1:10	0.65	+
36	Positive	+	1:20	0.92	-
37	Positive	-	1:10	0.56	+
38	None	-	-	0.13	-
39	Positive	+	1:40	0.65	+
40	None	+	-	0.44	+
41	nd	-	1:20	0.64	+
42	nd	+	1:10	0.36	+
43	nd	-	1:10	0.41	+
44	None	+	-	0.29	-
45	Positive	+	-	0.42	-
46	Positive	+	1:160	1.15	+
47	Positive	+	1:10	0.39	+
48	None	+	1:40	0.41	+
49	Positive	+	1:160	1.10	+
50	Positive	+	1:1280	1.31	+

TABLE A4. 1. (continued) FARM 2

SAMPLE NO.	LESIONS	LAT	CFT	ELISA	IBT
51	Positive	-	1:10	0.23	-
52	Positive	+	1:40	0.60	+
53	None	-	1:10	0.37	-
54	nd	+	1:40	1.12	+
55	nd	-	-	0.39	+
56	Positive	+	1:1280	1.25	+
57	None	+	1:1280	1.25	+
58	None	-	-	0.16	-
59	None	-	-	0.16	-
60	None	-	-	0.17	-
61	None	-	-	0.31	-
62	None	+	-	0.20	+
63	None	-	-	0.16	-
64	None	-	-	0.16	-
65	None	-	-	0.16	-
66	None	-	-	0.18	-
67	None	-	-	0.15	-
69	None	-	-	0.13	-
70	None	-	-	0.18	-
71	None	-	-	0.18	-
72	Positive	+	1:2560	1.10	+
73	Positive	+	1:2560	1.46	+
74	Positive	+	1:640	1.13	+
75	Positive	-	1:320	1.14	+
76	None	-	-	0.20	-
77	Positive	+	1:2560	1.28	+
78	Positive	+	1:10	1.11	+
79	None	-	-	0.27	+
80	Positive	+	1:160	0.62	+
81	Positive	+	1:320	0.75	+

TABLE A4. 1. (continued) FARM 3

SAMPLE NO.	LESIONS	LAT	CFT	ELISA	IBT
82	None	-	-	0.33	+
83	None	-	-	0.30	-
84	Positive	+	1:160	1.35	+
85	Positive	+	1:20	0.90	+
86	Positive	+	1:640	1.20	+
87	Positive	+	1:40	0.67	+
88	None	-	-	0.27	+
89	Positive	+	1:320	1.04	+
90	Positive	+	1:80	1.31	+
91	None	-	-	0.30	+
92	None	-	-	0.39	+
93	None	+	1:10	0.85	+
94	None	+	1:20	0.80	+
95	Positive	-	1:40	0.96	+
96	None	+	1:20	0.53	+
97	Positive	+	1:160	1.36	+
98	Positive	+	1:160	1.35	+
99	None	+	1:10	0.85	+

TABLE A4. 1. (continued) FARM 4

SAMPLE NO.	LESIONS	LAT	CFT	ELISA	IBT
100	None	-	1:80	0.83	+
101	None	-	-	0.20	-
102	None	-	-	0.15	+
103	None	-	1:20	0.19	+
104	None	-	-	0.19	-
105	None	-	-	0.18	-
106	nd	-	-	0.21	-
107	nd	-	-	0.06	-
108	nd	-	1:40	0.87	+
109	None	-	-	0.29	-
110	None	-	-	0.34	-
111	None	-	-	0.30	-
112	None	-	-	0.72	-
113	Positive	+	1:320	0.90	+
114	None	+	1:80	1.00	+
115	None	-	-	0.31	-
116	None	-	-	0.30	-
117	None	-	-	0.29	-
118	Positive	-	1:80	0.87	+

TABLE A4. 1. (continued) FARM 5

SAMPLE NO.	LESIONS	LAT	CFT	ELISA	IBT
134	nd	-	1:80	1.20	+
135	Positive	-	1:40	0.81	+
136	Positive	-	1:10	0.41	-
137	Positive	+	1:40	0.72	+
138	Positive	+	1:10	0.67	+
139	Positive	-	1:20	1.28	+
140	Positive	+	1:10	1.09	+
141	Positive	-	1:40	0.76	+
142	Positive	-	1:20	0.79	+
143	Positive	-	-	0.57	+
144	Positive	+	1:10	0.71	+
145	Positive	-	1:40	1.25	+
146	Positive	+	1:20	0.97	+
147	Positive	+	1:160	1.37	+
148	Positive	+	-	0.42	+
149	Positive	+	1:40	0.71	+
150	Positive	+	-	0.55	-
151	Positive	-	-	0.33	+
152	Positive	-	1:10	0.57	+
153	Positive	-	-	0.61	+
154	Positive	+	1:20	0.41	+
155	Positive	+	1:20	0.46	+
156	Positive	-	1:10	0.61	+
157	Positive	+	1:320	1.28	+
158	Positive	+	1:40	0.76	+
159	Positive	+	1:40	0.60	+
160	Positive	+	1:640	1.14	+
161	Positive	-	1:40	0.90	+
162	Positive	-	1:10	0.83	+
163	Positive	+	1:20	0.61	+
164	Positive	-	-	0.36	+
165	Positive	+	1:10	0.55	+
166	Positive	-	-	0.34	+
167	Positive	-	1:10	0.33	+
168	Positive	-	-	0.20	+
169	Positive	-	-	0.25	+
170	None	-	1:10	nd	+

TABLE A4. 1. (continued) FARM 6

SAMPLE NO.	LESIONS	LAT	CFT	ELISA	IBT
119	**Positive**	-	-	**0.38**	+
120	**Positive**	-	-	**0.38**	+
121	**Positive**	-	-	0.28	-
122	**Positive**	-	-	0.25	-
123	**Positive**	-	-	0.20	-
124	**Positive**	-	-	0.16	-
125	**Positive**	-	-	**0.33**	+
126	**Positive**	-	-	0.22	-
127	**Positive**	-	-	0.15	-
128	**Positive**	-	-	0.25	-
129	**Positive**	-	-	0.20	-
130	**Positive**	-	-	0.17	-
131	**Positive**	-	-	0.14	-
132	**Positive**	-	-	0.16	-
133	**Positive**	-	-	0.21	-

Results highlighted in bold are positive.

For the CFT a positive result in this study was considered to be >1:20.

nd = not determined.

For the ELISA the threshold value was considered to be 0.3; values of 0.25-0.29 were then scored as negative, though these tests might be considered as inconclusive. From the 6 farms only 9 samples fell in this range. For 3 samples all other tests were negative; 3 had lesions, one of which was also positive by IBT; 2 were only positive by IBT; and one was positive only by LAT.

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